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Biotechnic & Histochemistry

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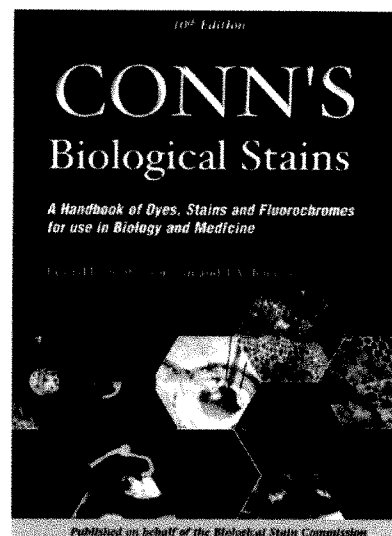
Conn's Biological Stains

A Handbook of Dyes, Stains and Fluorochromes for Use in Biology and Medicine

For 75 years *Conn's Biological Stains* has been a standard reference for all those who used dyes and colorants in the biological and medical sciences. This long awaited tenth edition appears 25 years after R.D. Lillie's ninth and has been completely rewritten to reflect the increase in range of uses. Although the staining of microscopical preparations continues to expand, the uses of dyes and fluorochromes now extend far beyond this traditional application.

This book provides the first critical overview of the whole range of low molecular weight fluorescent probes, outside the catalogue literature.

The first ten chapters are essays, by leading experts, on the important aspects of colorants and their uses. Most of the remainder of the book consists of descriptions by Dr Horobin of the properties and recent applications of hundreds of individual compounds, in about twenty chemical classes. The last chapter reviews the procedures employed at the Biological Stain Commission's laboratory to assay and test dyes and certify them as suitable for their intended applications.



R.W. Horobin and J.A. Kiernan (Eds)

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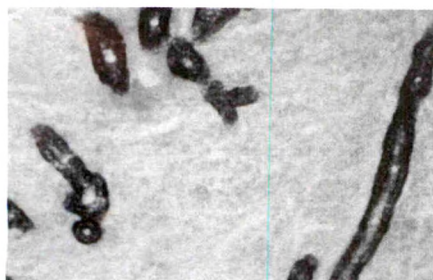
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Review of the 2002 Annual Meeting of the Biological Stain Commission

WE Grizzle, President

The Annual Meeting of the Biological Stain Commission (BSC) was held in Rockville, MD on June 7, 2002. The scientific session, organized by Dr. Robert S. Redman, focused on the methods for studying the morphology, development and pathology of salivary glands during "two turns of century," i.e., 19th-20th and 20th-21st.

Dr. Redman began the session with a general review of the morphology and functions of the major components of the salivary glands of rodents including acini, intercalated ducts, intralobular or striated ducts, and interlobular or excretory ducts. The importance of the secretions of salivary glands for maintaining teeth and healthy oral mucosa was emphasized using examples of the effects in man of the destruction of the salivary glands by Sjögren's Disease or by radiation therapy. Several pitfalls that can affect the study of rodent salivary glands were noted. He pointed out that the mostly mucous sublingual gland is within the same capsule as the serous/seromucous submandibular gland; hence there is a possibility that these two glands could be combined accidentally during experimental procedures if they are not separated adequately. Similarly, the diffuse location of the parotid gland in rodents could lead to portions of the parotid gland being confused or mixed with adjacent lacrimal glands. Another pitfall cited was the confusion by some investigators of the juxtaoral organ of Chievitz and the anlage of the parotid gland. Dr. Redman noted that the methods for PAS and alcian blue staining detailed by Dr. Robert Mowry were essential to investigations of salivary gland development and pathology.

Dr. Arthur A. Hand discussed molecular changes that occur during the development of the major salivary glands in rats. He reported that few changes have been identified in the molecular features of the sublingual glands as they develop. In contrast, both submandibular and parotid glands shift molecular patterns during development, the parotid gland changing at a somewhat later period during rodent development. He reported his study of a family of rodent peptides, SMG-A, BI, B2, C and

D, that he had identified earlier with Drs. Ball and Mirels. Although human correlates of these peptides have not been found to date, they are of special interest because there are shifts in their expression and localization during rat development. For example, the expression of SMG-B peptides shifts from the immature acini ("terminal tubules") of the perinatal submandibular gland to the intercalated ducts in the adult glands.

A presentation by Dr. Mathew Hoffman focused on the involvement of the fibroblastic growth factor family of growth factors and receptors in the development and branching of the mouse submandibular and sublingual salivary glands. His methods included identification of critically active genes in gland rudiments using microarray display, the preparation of anti-sense agents to block their expression, and a remarkable time-lapse video of their effects on glands in organ culture. He demonstrated that the interaction of FGF8, keratinocyte growth factor, with FGF receptor II was critical to controlling the cellular proliferation of the developing submandibular gland and its growth. FGF8 did not, however, control the branching of the submandibular gland. Dr. Hoffman also identified the importance of the alpha-3 component of laminin in the development of the mouse sublingual salivary gland.

Dr. Hans Lyon reported on Activities of the European Committee. He indicated that European standards for the information that should be included with biomedical diagnostic reagents have been approved by ISO (International Standards Organization). The standards are accompanied by four informative annexes including one for immunochemicals for the detection of estrogen receptor by immunohistochemistry and one for the detection of T cells by flow cytometry. The ISO standard will take effect after approval of the final draft of the International Standard. The ISO standard is expected to be in effect at the beginning of 2003. The standard will be revised in 2004.

Dr. Horobin presented a short discussion of the problems encountered by biologists, pathologists,

and biological researchers when describing biological staining, owing to their lack of training in chemistry. He especially emphasized that the terms "acid" and "basic" dyes are usually misnomers that should be replaced by the more specific terms, "cationic" and "anionic" dyes.

Dr. Richard Dapson of Anatech Ltd. reported a new staining procedure to replace the use of alcian yellow and toluidine blue for demonstrating *Helicobacter pylori*. Infection with *H. pylori* is thought to be the major cause of benign and malignant gastric ulcers.

Several administrative issues were discussed following the scientific sessions, including the success of the new design of *Biotechnic and Histochemistry* and the relatively smooth transfer of the journal to the new publisher, BIOS. A major advance for the BSC is that the new edition of Conn's Biological Stains is now available.

Annual certifications of dyes have declined from 1997 to 2001. The BSC also discussed continuing problems with primary antibodies and personnel issues related to immunohistochemical procedures. The BSC may re-evaluate its involvement in certification of immunohistochemical reagents. The BSC will begin a review of its goals and activities. This review will be conducted by the Charter

Committee, chaired by Dr. Grizzle and will be a major focus of the BSC during the 2002–2003 year.

The continuing education workshops presented at the 2002 annual meeting of the BSC were "How Dyes Stain Tissues", presented by Dr. Richard Dapson and "Interpretation of Special Stains", presented by Mr. Lamar Jones. Dr. A. Floyd, who organizes these continuing education workshops, reported that registrations for the continuing education workshops were disappointing.

The terms of Trustees William E. Grizzle, G. Stephen Nettleton, and David P. Penney expired in 2002, and by vote of the membership, the terms of these Trustees were extended to 2005. Dr. Robert S. Redman was elected as a new Trustee for 2002–2005.

The next meeting of the BSC will be June 5–7, 2003 in Rochester, NY. The scientific session will be organized by Dr. Richard Horobin. The 2004 meeting will be June 3–5, 2004 in Toronto, Canada, and Dr. Graeme Berlyn will organize the scientific session.

I hope all attending were pleased as I was with the quality of our 2002 meeting; however, I was disappointed with the attendance. I hope to see all members in Rochester, NY in 2003.

Microwave irradiation for rapid epidermis-dermis separation and improved epidermal cell immunodetection

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Abstract

We explored the effects of microwave irradiation on epidermal-dermal separation and subsequent immunostaining of epidermal cells. Epidermal sheets were obtained after incubation in 0.02 M EDTA in PBS and microwave irradiation with 4 pulses of 420 watts for 5 sec, with a total incubation period of 4 min. The control epidermal sheets were immunostained for Langerhans cells and dendritic epidermal T cells using a conventional immunoperoxidase method. The experimental immunodetection of these cells was assisted by incubating the primary antibodies for 10 min at 70 watts. We showed a simple and rapid method for separation of the epidermal-dermal junction and immunostaining of epidermal cells with optimal morphological preservation.

Key words: dendritic epidermal T cells, epidermal/dermal separation, immunohistology, Langerhans cells, microwave

The use of epidermal sheets in immunohistochemical procedures has allowed the characterization of epidermal cells, particularly in skin diseases where the epidermis plays an important role. To this end, epidermal-dermal separation is required for immunostaining procedures in large tissue samples. The epidermal-dermal separation procedures described in the literature are time-consuming, and on some occasions may cause significant tissue damage, thus complicating cell characterization.

Previously, we evaluated the efficacy of different epidermal-dermal separation procedures and concluded that the best separating agent was a solution of 0.02 M EDTA in phosphate-buffered saline (PBS), pH 7.3, compared to treatment with trypsin and thermolysin (Sánchez et al. 1990). The enzymatic treatments resulted in faster separation, but difficulties in removing the enzymes caused great damage to tissue integrity. In addition, although

EDTA solution allows good separation, the long incubation period, 150 min at 37° C, may contribute to normal degenerative processes.

Microwave irradiation has been widely used in tissue fixation (Chiu and Chan 1987, Azumi et al. 1990), and its success in immunocytochemistry may be due to the brief exposure of the tissue to the fixative, allowing better preservation of antigens.

Leong and Milios (1986) were the first to use microwave irradiation for immunocytochemistry to shorten the incubation period with primary antibodies recognizing T and B cell molecules in blood samples. Also, Chiu and Chan (1987) accelerated the three steps of immunoperoxidase staining in a microwave oven. In both cases, the results of subsequent experiments were inconsistent (Leong and Milios 1990).

The potential of immunostaining with microwave irradiation has been shown with a limited number of tissues and antigens. Takes et al. (1989) showed a substantial reduction in the total incubation time required for an immunoperoxidase procedure to detect lymphocyte antigens, neural proteins, intermediate filaments, muscle proteins and tissue enzymes in frozen and paraffin sections. Van Vlijmen and Van Erp (1993) enhanced the

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immunocytochemical signals to different cutaneous antigens in psoriasis lesions. These investigators and others proposed the use of different microwave incubation protocols for a distinct series of antibodies based on source and affinity of each particular antigen (Login and Dvorak 1992, Boon and Kok 1994, Marani 1998, Noyan et al. 2000).

In the present study, we used microwave irradiation to improve immunodetection of dendritic cells in separated epidermal sheets. The epidermal-dermal separation and immunoperoxidase staining incubation periods were substantially reduced, and gave excellent and consistent results.

Materials and methods

Epidermal separation

Skin biopsies from the hind footpads of BALB/c mice were cut into 1 mm² pieces. Each piece was immersed in a solution of 0.02 M EDTA in PBS, pH 7.3, in 15 × 45 mm (2 cm³) glass vials. Some samples were incubated under different conditions of microwave irradiation and others following a conventional incubation procedure at 37° C for 150 min. The irradiation protocol is summarized in Table 1. Briefly, pulses of microwave irradiation at different wattage (power percentages) were carried out using a microwave oven (EM-A 500T, Sanyo Electric Co., Osaka, Japan) with a maximum power of 700 watts. Between each pulse of irradiation, samples were incubated on ice.

After each schedule, the skin samples were washed in cold PBS, and the dermis was removed from the epidermis under a stereomicroscope using wooden toothpicks. The criterion for evaluating the epidermal separation was based on the ability to achieve a simple separation without tissue damage.

Monoclonal antibodies

Rat monoclonal antibody NLDC-145, which recognized the scavenger receptor DEC-205 on murine dendritic cells including epidermal Langerhans cells (Kraal et al. 1986), was diluted 1:30 (culture supernatant); a rat monoclonal antibody against Thy-1.2 (30H12), was diluted 1:50 (culture supernatant). Dilutions and immunostaining were carried out using PBS, pH 7.2. Both antibodies were kindly provided by George Kraal, Free University of Amsterdam, The Netherlands.

Immunoperoxidase staining

Immunoperoxidase staining was carried out as described previously (Sánchez et al. 1993) with some modifications for immunocytochemical characterization in epidermal sheets. Briefly, after fixation in fresh acetone for 5 min, the epidermal pieces were transferred to round-bottom microplates, hydrated in PBS, and incubated with primary rat monoclonal antibodies at room temperature for 90 min in the conventional protocol. In the microwave protocol, the microplates were placed in a humid chamber and tested at different incubation times (5, 10, 15, 20 and 30 min) in a microwave with 10% power (70 watts) and very short incubations (2 and 5 min) at medium level 50% power (350 watts). Two glass bottles filled with 100 ml ice-cold PBS were positioned in opposite corners of the microwave chamber as water loads. After primary antibody incubation, the rest of the procedure was carried out at room temperature using biotinylated rabbit anti-rat IgG (Vector Labs, CA) diluted at 1:30 (17 µg/ml) for 45 min, and avidin-biotin-horseradish peroxidase conjugate (Vectastain Elite, Vector Labs, CA) at 1:10 dilution

Table 1. Microwave irradiation schedules for dermal-epidermal separation

Power (%)	Watts	Pulses (no.)	Time/pulse (sec)	Total time (min)	Result*	Morphological preservation**
100	700	3	2	3	2	1
100	700	5	5	2	4	1
60	20	6	10	10	4	2
60	420	4	5	4	3	4
50	350	4	5	4	2	3
40	280	4	5	4	1	2
40	280	8	5	8	2	2
30	210	4	5	4	1	2
30	210	8	5	8	2	2
10	70	8	5	8	1	1

*Score for dermal-epidermal separation: 1, none; 2, difficult; 3, easy; 4, spontaneous.

**Score for morphological preservation: 1, poor; 2, moderate; 3, good; 4, optimal.

for 15 min. Five-minute washes with cold PBS were carried out between incubations. The reaction was developed for 5 min with 90 μ M H_2O_2 and 3-amino-9-ethyl-carbazole (AEC), final concentration 0.88 mM, which was dissolved in 50 mM N,N-dimethylformamide in 0.1 M acetate buffer, pH 5.2. The epidermal sheets then were washed and mounted on glass slides with glycerin-gelatin. Controls consisted of omission of the primary antibody or use of an antibody of irrelevant specificity at the same concentration.

Results

The results of the epidermal separation are summarized in Table 1. Rapid separations were obtained with microwave pulses at high power (420–700 watts), but the heat generated caused significant tissue damage. No separation was obtained at low power irradiation (70–320 watts). The best result was found with 420 watts (60% power) using four pulses of 5 sec each and intervals of 60 sec at 4° C with a total incubation period of 4 min instead of the 150 min used in the conventional procedure. With this schedule, the epidermal separation was easier and the morphology better preserved than in the conventional method.

Once we tested different incubation times at low power microwave irradiation to avoid hot spots and their effect on the yield of antigen-antibody complexes, best immunohistologic results were obtained with incubations of 10 min for the primary antibodies, thus reducing the immunostaining period by 89%.

Epidermal sheets immunostained with microwave-assisted primary antibody incubation showed similar cell proportions, but the morphology of immunoreactive cells was preserved better than in conventionally stained tissue (Fig. 1).

Ten-minute microwave irradiations reduced the incubation time without affecting the formation of antigen-antibody complexes, whereas tissues subjected to higher incubation times (15, 20 and 30 min) showed strong and diffuse background and weakly immunostained epidermal cells (Fig. 2). Sheets incubated for a short time at 350 watts (50% power) showed weak immunostaining compared to non-microwaved control tissue (data not shown).

Discussion

Although microwave irradiation has been widely used for tissue fixation and immunostaining procedures, care must be taken to avoid damage to the tissue integrity. Hjerpe et al. (1988) showed that the

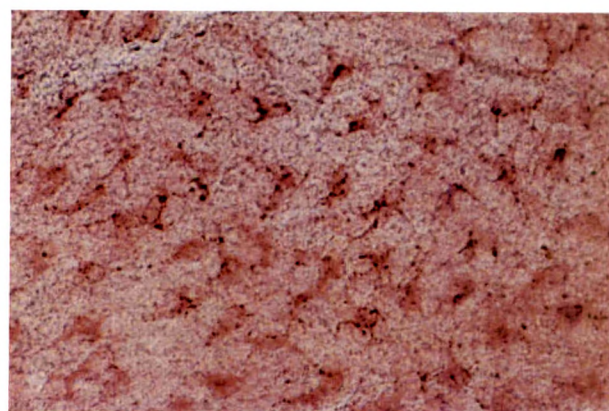
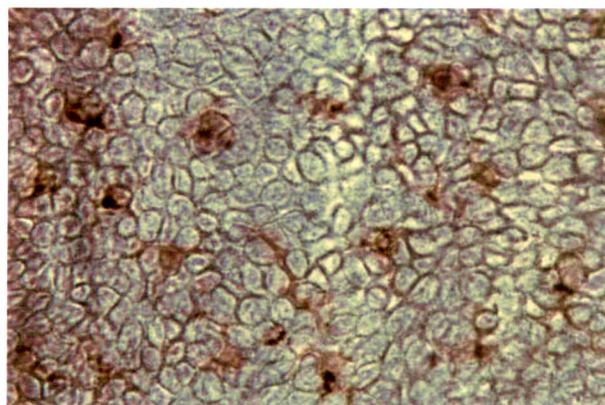


Fig. 1. Detection of DEC-205 positive Langerhans cells on separated epidermis. A) Microwave-assisted epidermal-dermal EDTA separation at 420 watts with 4 pulses of 5 sec during 4 min, and primary antibody incubation at 70 watts for 10 min. $\times 1000$. B) Conventional epidermal-dermal EDTA separation for 150 min, and primary antibody incubation for 60 min. $\times 1000$.

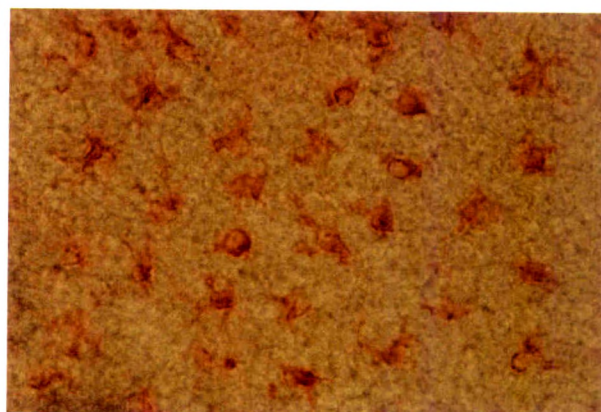


Fig. 2. Detection of DEC-205 positive Langerhans cells on EDTA-separated epidermis. Microwave-assisted epidermal-dermal separation at 420 watts with 4 pulses of 5 sec during 4 min, and primary antibody incubation at 70 watts for 20 min. $\times 1000$.

temperature in the droplets must be controlled to avoid reduction in the formation of antigen-antibody complexes. Moreover, Yasuda et al. (1990) demonstrated that 2 ml PBS water loads increase the temperature by 10° C in 10 sec microwave irradiation at 700 watts (100% power). For that reason, we used irradiation pulses of 5 sec at 420 watts with cooling on ice between each pulse to avoid excessive temperature increase and to favor optimal epidermis separation. This procedure allowed epidermis-dermis separation in only 4 min instead of the 150 min incubation required for the conventional method. The results also showed that 4 min microwave-assisted EDTA rapidly and efficiently disrupted the hemidesmosomal junctions between dermis and epidermis, but that additional incubation time may affect keratinocyte tight junctions, causing severe tissue damage.

The reproducibility of the present microwave-assisted immunocytochemical method for the characterization of epidermal cells was successfully achieved by carrying out the incubations with the secondary biotinylated antibody and the avidin-biotin-peroxidase complex outside the microwave oven to maintain the stability of the reactants.

In the present study, microwave-stimulated immunocytochemistry may favor accelerated diffusion of antibodies through agitation during the incubation time, thus improving the sensitivity of the method. Although the microwave action mode is still unknown, evidence shows that the rapid oscillation of dipolar molecules (i.e., water) and polar molecules (i.e., proteins) causes molecular collisions and acceleration of the chemical reaction rate (Yasuda et al. 1990). In addition, the heat generated by microwave irradiation can contribute to the increase in the reaction rate, but it can also damage biological components such as proteins, antibodies, antigens, and even the general structure of the tissue. For that reason, we used water loads containing the incubation buffer to diminish the heat inside the sample during the extended irradiation step.

We also tested microwave-stimulated secondary fluoresceinated antibody, and obtained appropriate immunostaining of Langerhans cells using 210 watts and irradiation for 10 min with no decrease in the fluorescence stability compared to the conventional indirect immunofluorescence procedure (data not shown).

The microwave device used in the present study is a conventional household oven, not a laboratory microwave with accurate temperature control. Short microwave pulses at specific wattage and appropriate water loads ensure reproducible and

successful epidermis-dermis separation and improved epidermal cell immunostaining.

Microwave irradiation is useful for reducing the incubation time for epidermal separation and immunohistology, improving immunostaining detection, and maintaining tissue integrity in separated epidermis.

Acknowledgments

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An enzyme histochemical and biochemical study of the activity of three oxidative enzymes in the developing rat parotid gland

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Abstract

Information on ductal differentiation in the developing rat parotid gland is sparse. One of the main functions of the striated and excretory ducts in this gland is the selective exchange of electrolytes from the primary fluid secreted by the acini. These ducts are rich in a number of enzymes involved in this task, suggesting that they might be useful as markers of ductal differentiation. The objective of this investigation was to delineate the developmental changes in activity of three of these, cytochrome C oxidase (CCO), succinate dehydrogenase (SDH), nicotinamide adenine phosphate dinucleotide (reduced form)-dehydrogenase (NADPH-DH). Histochemical localization of all three enzymes in fresh frozen sections was complemented by biochemical assays of CCO and SDH and cytochemical localization of CCO. Biochemically, CCO- and SDH-specific activity in gland homogenates increased progressively after birth, reaching adult levels at 21–28 days. Histochemically, deposits of reaction products of all three enzymes increased more in the striated and excretory ducts, especially in their basal cytoplasm, than in other glandular structures between 19 days *in utero* and 28 days after birth. During the same age span, the mitochondria in the striated and excretory ducts increased markedly in both number and size, migrated to a mostly basal location, and increased from many to virtually all showing strong cytochemical CCO reactions. These histochemical and cytochemical patterns of changes in enzyme activity at the cellular level accounted for the overall increases in CCO and SDH seen in the biochemical assays. Only the SDH histochemical reaction was consistently weak in the acini and intercalated ducts, and thus provided the most contrast with the progressively stronger reactions in the larger ducts. We conclude that of the three enzymes evaluated in these experiments, SDH is the best marker of the functional differentiation of the striated and excretory ducts in the developing rat parotid gland.

Key words: cytochrome C oxidase, development, differentiation, NADPH dehydrogenase, parotid gland, rat, salivary gland, succinate dehydrogenase

Extensive research on the development of the salivary glands of rats has occurred during the past 40 years (reviews: Redman 1987, 1994; Denny et al. 1997). Most of these studies have described either morphologic development through the use of light and electron microscopy, or functional measures of

development through analysis of gland homogenates, saliva, or secretory products obtained by micropuncture. In addition, these tools have been used to examine alterations within the gland as variables such as autonomic innervation, genes, hormones, nutritional status, circadian cycles and pharmacologic agents have been monitored or manipulated. Histochemistry also has found considerable use in localizing the glandular and intracellular distribution of electrolytes and secretory products, and the enzymes involved in their synthesis and transport in these glands.

While histochemistry has been employed to study developing salivary glands of the rat, it has

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been used mostly to describe the secretory components (acini and, in submandibular glands, the convoluted granular tubules) and the myoepithelium. In particular, work on the development of the ductal system of the rat parotid gland has been sketchy. The recent development of methods of primary culture of cells (Durban 1990, Yeh et al. 1991, Quissell et al. 1994) and of cell lines (Quissell et al. 1998) from rat salivary glands has raised the need for markers of the functional differentiation of the various glandular components. Although these seem ample for the acini and myoepithelium, they have been inadequately explored for the striated and excretory ducts.

The purposes of this project, therefore, were to: (a) document the developmental chronology of the activity of some of the enzymes reported to have higher activity in the larger ducts than in other parenchymal elements of the rat parotid gland; (2) link these enzymes to specific intracellular locations; and (3) from the results, determine which of these enzymes may best serve as markers of differentiation of the striated and excretory ducts of the gland. In previous reports, we used enzyme histochemistry for nicotinamide adenine dinucleotide dehydrogenase (NADH-DH) and ouabain-sensitive Na^+, K^+ -adenosine triphosphatase (Na^+, K^+ -ATPase; Peagler and Redman 1999), and both enzyme- and immunohistochemistry to localize carbonic anhydrase (CA; Peagler et al. 1998).

We report here the last three of the enzymes that were selected for histochemical analysis: cytochrome C oxidase (1.9.3.1, CCO), succinate dehydrogenase (1.3.99.1, SDH), and nicotinamide adenine phosphate dinucleotide (reduced form)-dehydrogenase (NADPH-DH). Previous enzyme histochemistry by others has demonstrated that these are localized most strongly in the ducts of rodent salivary glands (Bordeianu et al. 1983, Kawakatsu et al. 1962, Shapiro 1967). The first two of these enzymes reside primarily in mitochondria (Bertoni-Freddari et al. 2001) which are concentrated along the basolateral infoldings and interdigitations of the striated and excretory ducts in mature rat parotid glands (Hand 1987). It is apparent that the mitochondria provide the large amounts of energy required for one of the principal functions of these ducts, the selective reabsorption and secretion of ions from the primary fluid. Reaction product for the NADPH-DH procedure is concentrated not only in the basolateral cytoplasm (presumably associated with the mitochondria), but also in or subjacent to the luminal membranes of the striated and excretory ducts (Shapiro 1967). It was anticipated that the patterns

of these enzymes in the developing parotid gland would provide valuable information about both the structural and functional differentiation of these ducts. Biochemical assays were performed for CCO and SDH in gland homogenates to provide a complementary overview. In addition, it had been noticed in a previous study on salivary peroxidase in the developing rat parotid gland (Redman and Field 1993) that electron micrographs showing cytochemically localized peroxidase activity in the secretory granules and RER also demonstrated CCO activity in the mitochondria. Subsequently, it has been reported that differences in density of cytochemical reaction products of CCO and SDH can be related to the functional competence of these mitochondrial enzymes (Bertoni-Freddari et al. 2001). As similar variations in CCO reaction product seemed to be present in the mitochondria in our electron micrographs, an analysis of these was added to the study.

Materials and methods

Experimental animals

Sprague-Dawley rats (*Rattus norvegicus albinus*) were obtained from colonies maintained either at Howard University or in the Research Animal Facility of this VA Medical Center, with breeders purchased viral pathogen-free from Charles River, Wilmington, MA. This assured a high degree of accuracy in the ages sampled. *In utero* (i.u.) ages began with 0 on the day when vaginal plugs were observed, and postnatal ages began with 0 on the day of birth. Dams were housed as soon as pregnant in individual nest boxes in a room with controlled temperature and humidity, and lights on at 0600 h and off at 1800 h daily. Pups were weaned at age 21 days and housed by litter in large cages. All animals were allowed unlimited access to water and a commercial pelleted diet. These and all other aspects of the care and use of the animals were according to U.S. Department of Agriculture standards, and all experimental procedures had the prior approval of the Research and Development Committee of the Washington, DC, VA Medical Center.

Samples were obtained for the biochemical assays on days 1, 7, 14, 21, 28 and 42 after birth. The same animals were shared for the two assays. Glands were used from four males and four females at each age ($n = 8$), except that at ages 1 and 7 days, homogenates from four litters of eight or more pups each were pooled by litter ($n = 4$).

For the histochemical procedures, the following ages were selected for analysis: 19 days *i.u.* and 0, 1, 7, 14, 21, 28 and 40 days after birth. Equal numbers of male and female rats were obtained from at least two different litters for each age (minimum $n = 4$). To obtain sufficient tissue, glands were pooled by litter at the perinatal (19 days *i.u.* - 1 day postnatal) ages, and by sex (2 rats each from each litter) at 7 days. Tissues were not pooled at ages 14 days and older.

The rats were weighed, then killed by cervical section. The parotid glands were excised and freed of extraneous tissues with the aid of a dissection microscope. For prenatal samples, dams were anesthetized with pentobarbital sodium (Abbot Laboratories, North Chicago, IL; 50 mg/kg body weight, *i.p.*) prior to removal of their offspring, immediately after which they were exsanguinated by cardiac puncture.

Biochemical assays

All chemicals used for the biochemical and histochemical procedures were reagent grade and, except as noted, obtained from Sigma Chemical Co., St. Louis, MO.

The freshly excised and cleaned glands were quick-frozen in capped glass vials on dry ice and kept at -70°C . Just before assay, glands were thawed with a gentle stream of cold water onto the vials, placed in vessels on ice, homogenized with an ultrasound probe, and divided into aliquots. Aliquots not used immediately were frozen and stored at -70°C . Each assay was performed in triplicate, with the average used as the "best estimate" for that sample. Each run consisted of samples across the age spectrum.

CCO was assayed by the macromethod of Cooperstein and Lazarow (1951), with cytochrome C as the substrate. The rate of extinction at 550 m μ was determined during continuous stirring in the cuvette in a Shimadzu 160 UV spectrophotometer modified for this purpose as previously described (Redman and Field 1993).

SDH was assayed by the method of Pennington (1961) with sodium succinate as the substrate and 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (*INT*) as the indicator. The resulting formazan was extracted and its extinction measured at 490 m μ in the Shimadzu spectrophotometer.

Protein in the homogenates was determined by the method of Lowry et al. (1951) using bovine serum albumin (fraction V) as the standard.

The results of the enzyme assays are expressed as activity per milligram of protein (specific activity).

For both assays (CCO and SDH), preliminary tests showed no significant difference in activity between fresh and frozen aliquots from the same samples.

Histochemical procedures

Gland samples were quick-frozen by immersion in the viscous phase of isopentane (4-methylbutane) in a test tube bathed in liquid nitrogen. This minimized damage to the tissue by limiting the growth of individual ice crystals. Frozen samples were stored immersed in liquid nitrogen until mounting on a cryostat block with O.C.T. compound (Miles Scientific, Naperville, IL) with care not to thaw the tissues during transfer and mounting. Sections approximately 12 μm thick were cut at -20°C , except for the perinatal samples, which sectioned better at -14°C . Sections were thaw-mounted on 5% gelatin-coated slides and air dried for 20 min to attach them to the slides and fix the tissue prior to staining. After appropriate incubation for each enzyme, sections were washed with tap water for 1 min, immersed in 70% ethanol for 2 min, counterstained with eosin, immersed for 2 min each through two changes of 90 and 100% ethanol and xylol, and coverslipped with Pro-Texx[®] (Lerner Laboratories, Pittsburgh, PA). No visible reaction occurred with any of the enzymes without the respective substrates. If a weak reaction occurred at the younger ages, alternate slides were not counterstained to visualize better the density and localization of the reaction product.

CCO was localized in the sections as described by Troyer (1980), as adapted from the method of Seligman et al. (1968), with cytochrome C as the substrate and 3,3'-diaminobenzidine HCl (DAB) as the chromogen. Incubation for 40 min at 37°C provided optimal density and localization of the reaction product in trials with sections of glands from adult (70 days old) rats.

SDH and NADPH-DH were localized as described by Troyer (1980). For both enzymes, nitroblue tetrazolium (Nitro-BT) was the chromogen, the optimum concentration of MgCl_2 was 0.084 M, and the optimum incubation was for 20 min at 37°C . For SDH, the substrate was sodium succinate. No intermediate electron carrier was added to the medium, because a strong reaction occurred in sections of glands from adult rats without one. The substrate for NADPH-DH was NADPH.

Two of us (JSJ, RSR) subjectively assessed the intensity of each reaction by age and cellular location, then arrived at a consensus with a score of 0 indicating no precipitate and 5 indicating the heaviest obtained.

Cytochemical procedures

These were as described previously (Redman and Field 1993). Except as noted, all procedures were at ambient temperature (*ca.* 23° C). Briefly, 1 × 1 × 2 mm blocks of gland were immersed in phosphate-buffered 3.0% formaldehyde (freshly prepared from paraformaldehyde) for 2 h, rinsed thoroughly in the phosphate buffer plus sucrose, immersed in tris-HCl buffer with pre-mixed DAB and H₂O₂ for 2 h at 37° C, rinsed again, postfixed in 1.0% OsO₄, and processed and embedded (final incubation at 60° C) in epoxy resin. Most sections for light and electron microscopy were viewed and photographed without additional staining.

Statistical analysis

The results of the biochemical assays were compared for overall differences by analysis of variance (ANOVA) and by age with Duncan's multiple range test (Snedecor and Cochran 1980). Differences were considered significant at $P < 0.05$. Despite

our best efforts to be objective in the histochemical scoring, these scores were not compared statistically, for to do so would imply a greater precision than can be achieved with the method used.

Results

Experimental animals

The increases in body and gland weights with age were similar to those reported in a previous study (Redman and Field 1993), and indicate uniformly good growth and health.

Biochemical assays

No significant differences were found in the specific activity of either CCO or SDH between male and female samples at any age. Therefore, the combined (male + female) data are presented. Both CCO (Fig. 1) and SDH (Fig. 2) showed progressive increases in specific activity between birth and 21–28 days.

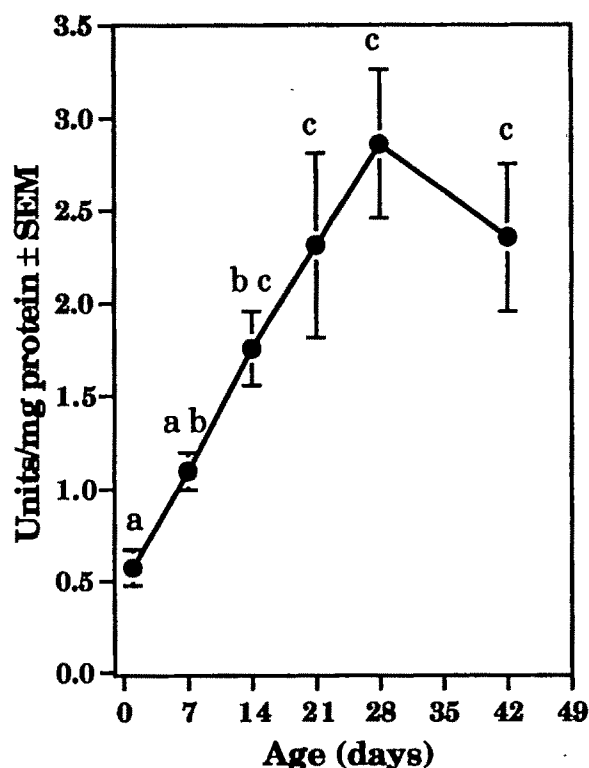


Fig. 1. Cytochrome C oxidase specific activity (mean $\pm$ SEM) in parotid gland homogenates at representative postnatal ages. Glands were used from at least four males and four females at each age ($n = 8$), except that at 1 and 7 days, homogenates from four litters of pups were pooled by litter ($n = 4$). Values not sharing the same superscript differ significantly ($P < 0.05$).

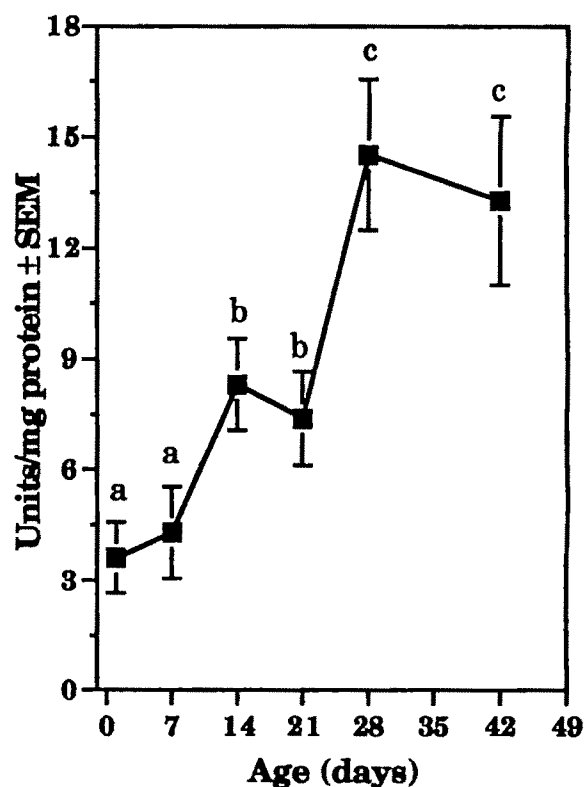


Fig. 2. Succinate dehydrogenase specific activity (mean $\pm$ SEM) in parotid gland homogenates at representative postnatal ages. Samples for this assay were taken from the gland homogenates that were used for the cytochrome C oxidase assays. Values not sharing the same superscript differ significantly ($P < 0.05$).

Histochemistry

Photomicrographs of the histochemically localized enzyme reactions at representative ages are shown in Figures 3–5, and a compilation of the histochemical scores is presented in Table 1.

CCO activity (Fig. 3) was moderate in the ducts at 19 days *i.u.* and gradually increased with the strongest levels being concentrated in and along the basal cytoplasm and membranes of the striated and excretory ducts by 21 days. Activity in the luminal membranes plateaued after 1 day after birth. Activity was modest within the terminal buds and acini until 1 day, then increased somewhat to the moderate levels observed in the adult gland.

SDH activity (Fig. 4) at age 19 days *i.u.* was weak and homogeneously distributed among the relatively undifferentiated cells of the ducts and

terminal buds. After birth, the activity increased slightly to the still modest levels of adult rats in the terminal buds and small ducts as they differentiated into acini and intercalated ducts. In contrast, the large ducts, as they differentiated into striated and excretory ducts, showed a gradual increase in SDH activity until 14 days. At that point the basolateral aspects of these ducts began to show a marked increase in SDH activity until the pattern of the mature gland was achieved at 28 days and older.

NADPH-DH activity (Fig. 5) at 19 days *i.u.* was already moderate in the ducts and terminal buds, then gradually increased in the developing striated and excretory ducts, with reaction product becoming increasingly concentrated along both basal and luminal membranes after birth. Activity remained moderate in the acini, but increased somewhat in the intercalated ducts after birth.

Table 1. Histochemical assessment of the activity of three oxidative enzymes in the developing rat parotid gland

a. Cytochrome C oxidase

Age (days)	Acini	Ducts		
		Intercalated	Striated	Excretory
19 <i>i.u.</i> *	1	----- 1 -----		1
0	1	1	1 B, 2 L	1 B, 2 L
1	1	1	2 B, 3 L	3 B, 2 L
7	2	2	3 B, 2 L	3 B, 2 L
14	2	2	3 B, 2 L	3 B, 2 L
21	3	2	3 B, 2 L	4 B, 2 L
28	3	2	4 B, 2 L	4 B, 2 L
40	3	2	5 B, 2 L	5 B, 2 L

b. Succinate dehydrogenase

19 <i>i.u.</i> *	1	----- 1 -----		1 B, 1 L
0	1	1	1 B, 1 L	1 B, 1 L
1	1	1	1 B, 2 L	1 B, 2 L
7	1	1	1 B, 2 L	1 B, 2 L
14	1	1	2 B	2 B
21	1	1	3 B	4 B
28	1	1	5 B	5 B
40	1	1	5 B	5 B

c. Nicotinamide adenine dinucleotide phosphate (reduced form) dehydrogenase

19 <i>i.u.</i> *	2	----- 2 -----		2 B, 3 L
0	2	2 B, 2 L	2 B, 3 L	3 B, 3 L
1	2	2 B, 2 L	2 B, 3 L	3 B, 3 L
7	2 B	2 B, 3 L	2 B, 3 L	3 B, 4 L
14	2 B	2 B, 3 L	2 B, 3 L	3 B, 4 L
21	2 B	2 B, 3 L	3 B, 4 L	4 B, 4 L
28	2 B	2 B, 3 L	4 B, 5 L	5 B, 5 L
40	2 B	2 B, 3 L	4 B, 5 L	5 B, 5 L

* *In utero*. Scores ranged from 0 (none) to 5 (heaviest precipitate of reaction product). Letters refer to locally heavier precipitates within structures: B, basal; L, luminal. At 19 days *i.u.*, intercalated and striated ducts were insufficiently differentiated to distinguish reliably and so share one score.

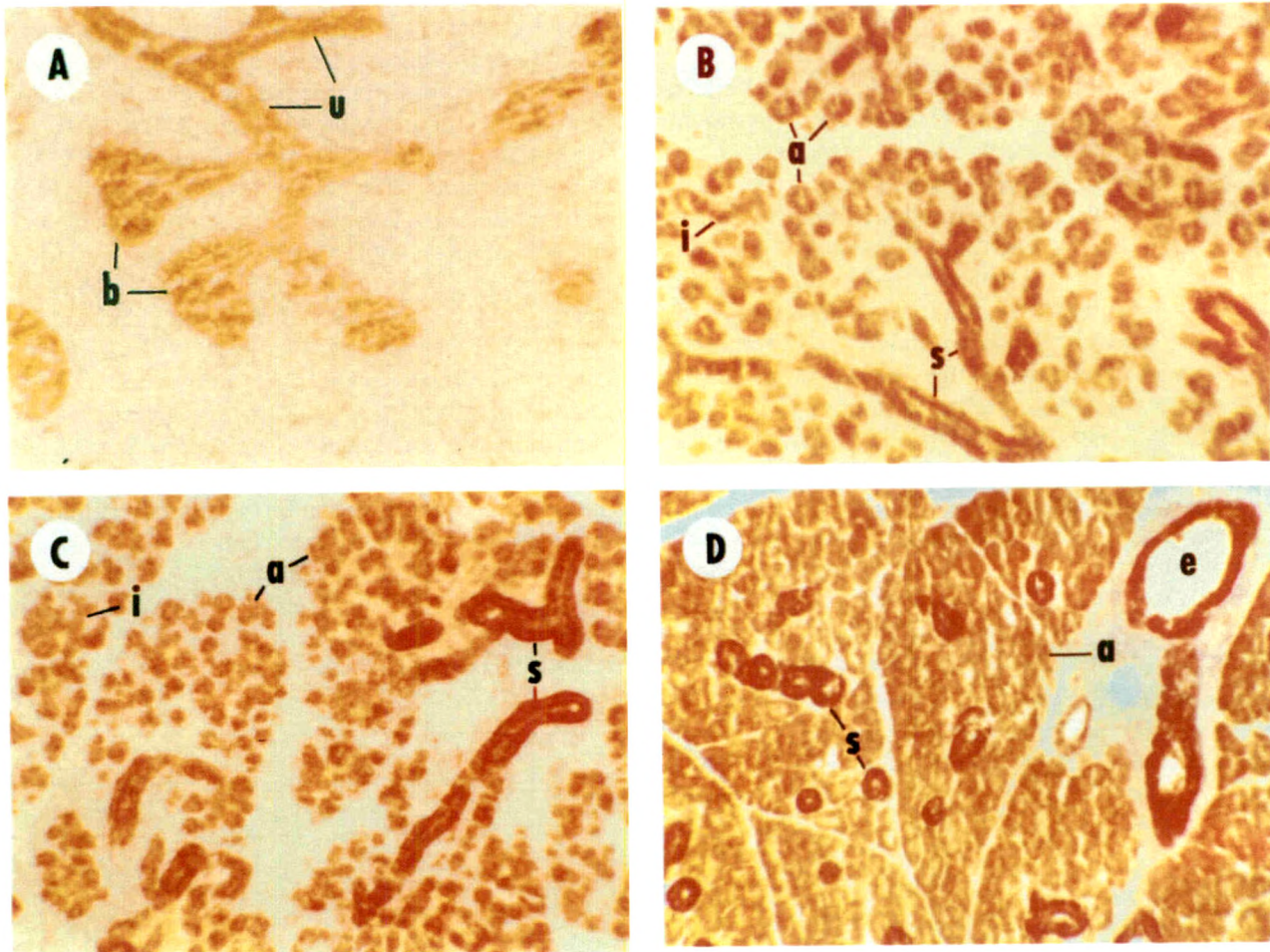


Fig. 3. Enzyme histochemically localized cytochrome C oxidase activity in the developing rat parotid gland at ages (A) 19 days *in utero*, and (B, C, D) 7, 14 and 40 days after birth. a = acini; b = terminal buds; e, i, s, u = excretory, intercalated, striated and undifferentiated ducts. Cytochrome C substrate, DAB chromogen, eosin counterstain. All $\times 110$.

Cytochemistry

Deposits of reaction product on the cristae of mitochondria, and thus attributable to CCO and not peroxidase, occurred in all parenchymal components at all ages (Figs. 6–8). The density of the deposits varied considerably among mitochondria in the terminal buds (Fig. 6A), mature acini, intercalated ducts and myoepithelium (Fig. 6B), and in the luminal cytoplasm in both immature (Fig. 7A,B) and mature (Fig. 8) striated ducts. The density, however, was almost uniformly heavy in the mitochondria of the basal cytoplasm of the striated ducts at all stages of differentiation. Striated duct differentiation was characterized by an increase in the size of the cells from short to tall columnar, a marked increase in the size and number of mitochondria, and a shift in the distribution of mitochondria from about half each in the luminal and basal cytoplasm to predominantly basal. Thus,

virtually all mitochondria in well-differentiated striated ducts demonstrated heavy CCO activity. The developmental pattern in the excretory ducts tended to be similar to that of the striated ducts (not illustrated).

Discussion

Correlation of results

There was good correlation among the biochemical, histochemical and cytochemical observations on CCO and SDH. All demonstrated substantial increases in activity after birth and achievement of adult levels and patterns by 28 days. The histochemical and cytochemical localization showed that the overall increase recorded by the biochemical assays was due to the sum of several components. The most important of these was the maturation of the large ducts, whose mitochondria increased

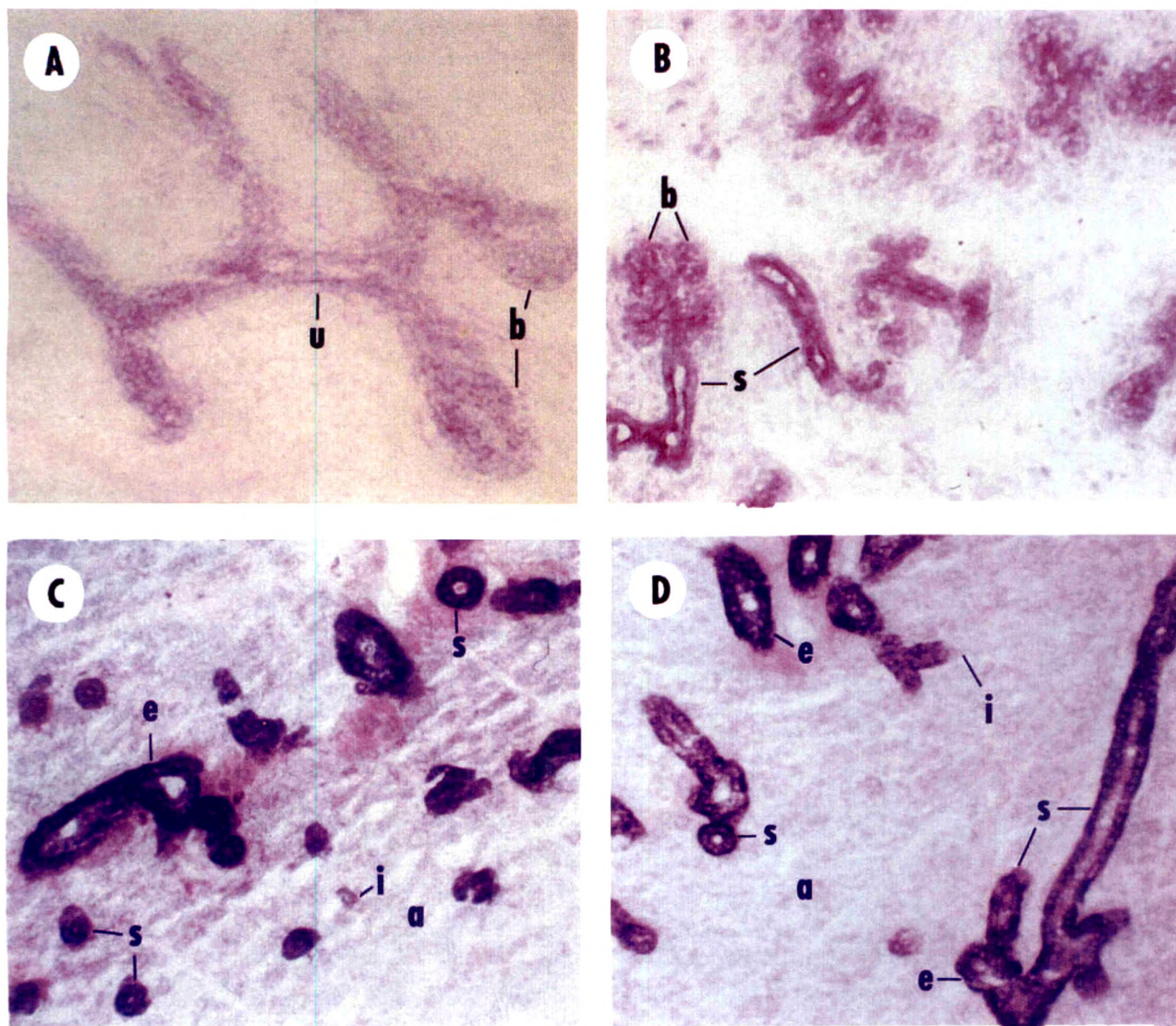


Fig. 4. Enzyme histochemically localized succinate dehydrogenase activity in the developing rat parotid gland at ages (A) 19 days *in utero*, and (B, C, D) 1, 28 and 40 days after birth. a = acini; b = terminal buds; e, i, s, u = excretory, intercalated, striated and undifferentiated ducts. Sodium succinate substrate, NTB chromogen, eosin counterstain. All $\times 140$.

dramatically in number and size, migrated basally, and developed uniformly strong CCO activity. In addition, as noted in this and previous studies (Redman 1987), the proportion of gland occupied by protein-rich but mitochondria-poor stroma shrank from large during the perinatal period to small by 18 days after birth. This happens as a result of the parenchymal cells, especially the acini, proliferating and enlarging to near confluence except for sheaths around the largest ducts. This undoubtedly accounts for some of the observed overall increase in the specific activities of CCO and SDH despite only slight postnatal increases in their histochemically localized reactions in the acini. The latter appears to be due to the displacement of the mitochondria by the large accumulation of the

machinery for synthesis and storage of secretory materials, i.e., the rough endoplasmic reticulum, Golgi apparatus and secretory granules, as the acini matured. This tended to dilute the density of reactions in individual acini despite any increase in mitochondrial number or size that might have occurred with maturation.

Comparisons with other studies

Ultrastructurally, striated ducts in the developing rat parotid gland have been reported to be recognizable by 7 days and almost mature by 20 days (Taga and Sesso 1979). Micropuncture studies indicate that the electrolyte resorptive capacities of the striated and excretory ducts are mature by 18

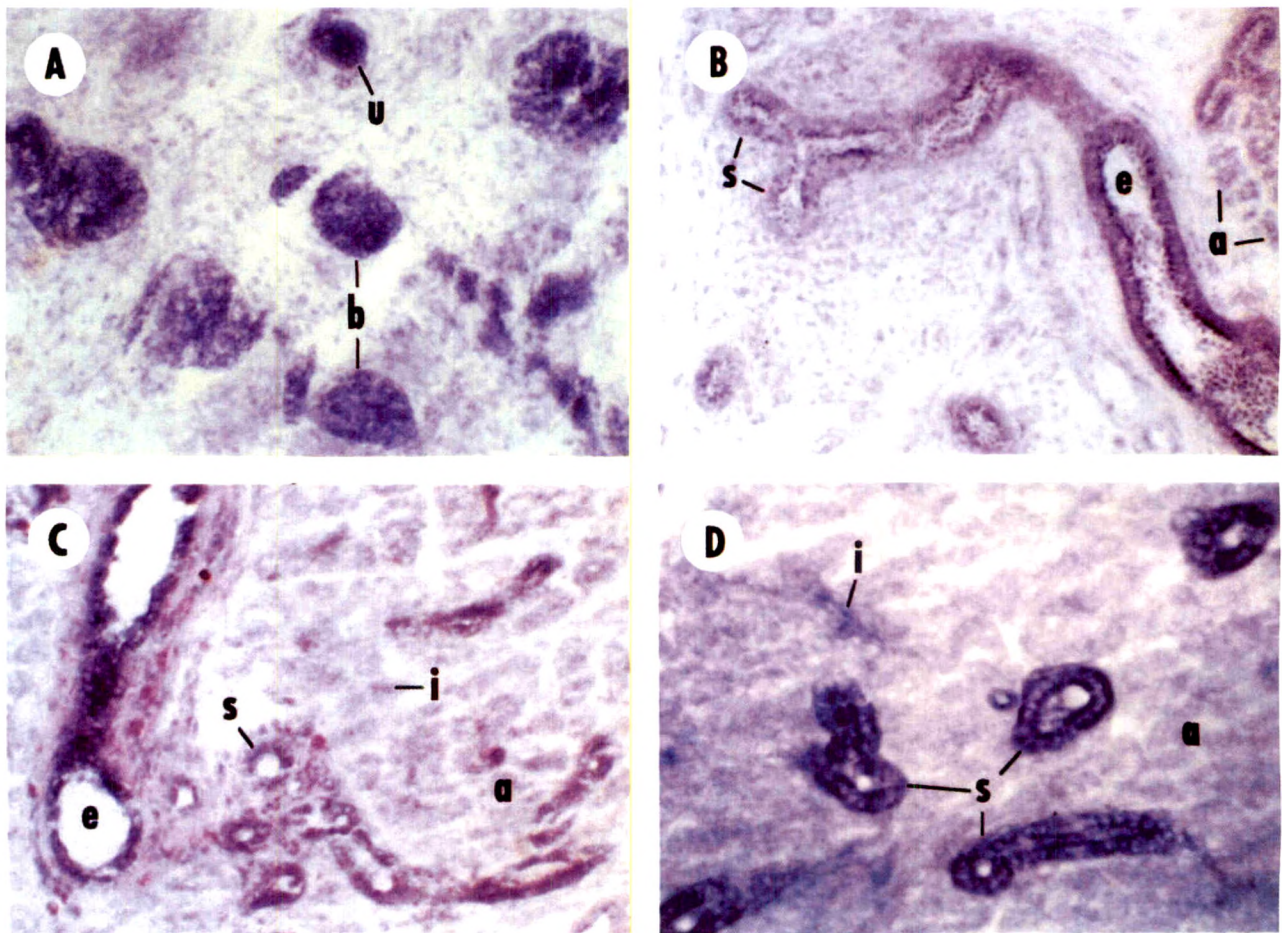


Fig. 5. Enzyme histochemically localized NADPH dehydrogenase activity in the developing rat parotid gland at ages (A) 19 days *in utero*, and (B, C, D) 14, 21 and 40 days after birth. a = acini; b = terminal buds; e, i, s, u = excretory, intercalated, striated and undifferentiated ducts. NADPH substrate, NTB chromogen, eosin counterstain. A, B, C $\times 140$; D $\times 275$.

days (Mangos 1978). The electrolyte pattern of stimulated saliva, however, does not approach that of the adult until 30 days (Schneyer and Schneyer 1961, Schneyer and Hall 1968).

In general, the postnatal increases in the activities of SDH and CCO parallel the accumulation of mitochondria in the basal parts of the striated and excretory ducts reported by Taga and Sesso (1979). Ultrastructurally, however, we observed a further increment of mitochondrial accumulation between 21 and 28 days. This better matches the pattern of increases in CCO and SDH presented here, and of CA (Peagler et al. 1998, Ogawa et al. 1998) and Na^+, K^+ -ATPase (Peagler and Redman 1999) reported previously.

Moderate luminal and basal activity of NADPH-DH was observed in the larger ducts of glands from perinatal rats. As this precedes the accumulation of large numbers of mitochondria in these structures, much of the activity of these enzymes apparently resides in or near the luminal and basolateral

portions of the plasmalemma at this stage of gland development.

Markers of ductal differentiation

All three enzymes were localized histochemically more strongly in the striated and excretory ducts of mature rat parotid glands. Only the SDH reaction, however, was weak enough in the terminal buds, acini and intercalated ducts at all ages to be useful for distinguishing cells of the larger ducts in cell culture, where recognition is not facilitated by position among other parenchymal structures (acini, intercalated ducts, myoepithelium and stroma). We conclude that, of the enzyme histochemical procedures examined in our investigations, those for SDH, Na^+, K^+ -ATPase and CA should be the most useful as markers of differentiation of striated and excretory ducts, both in parotid gland cells in culture and in sections of parotid glands of young and adult rats.

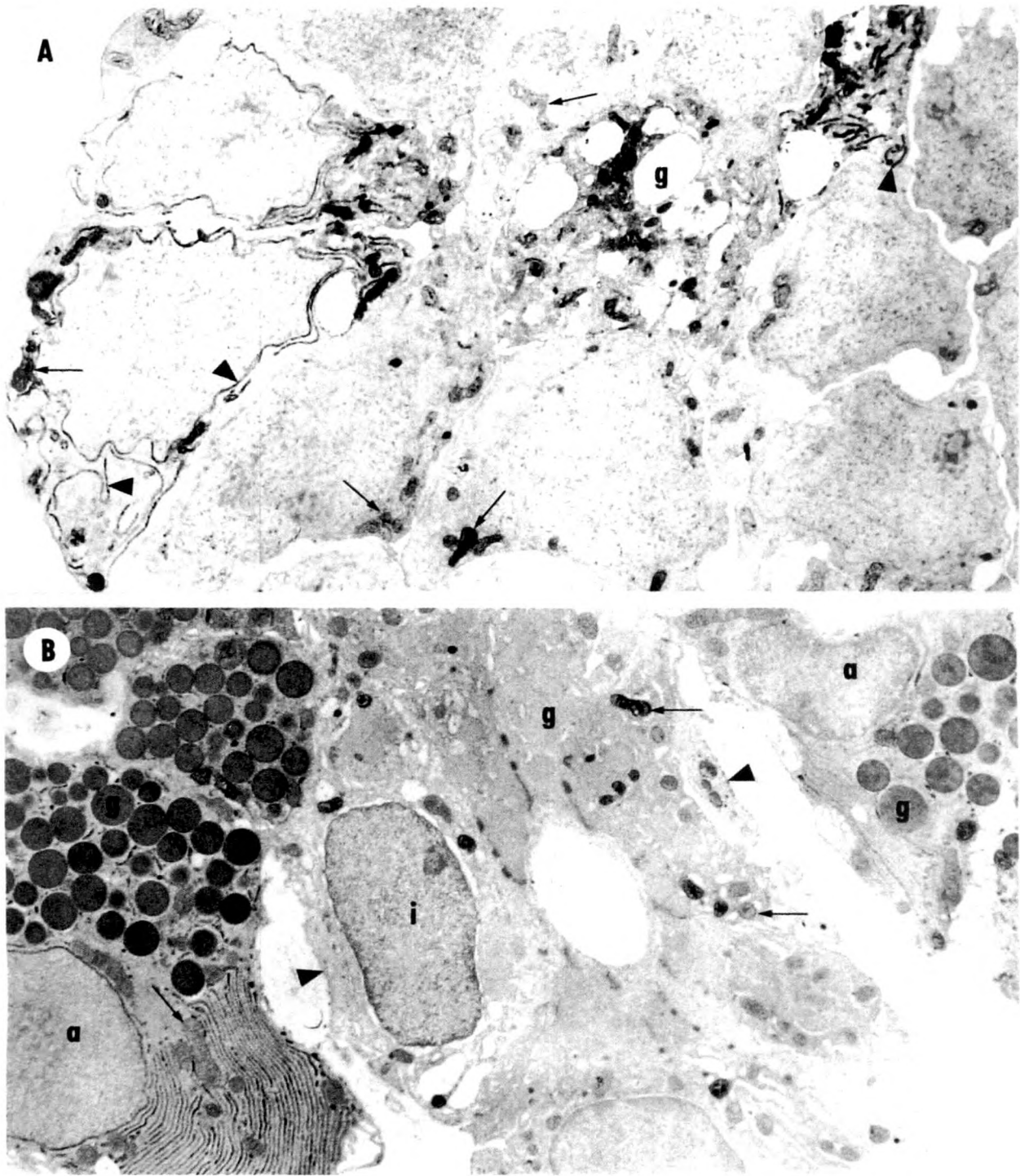


Fig. 6. This and the following illustrations are transmission electron micrographs of rat parotid glands prepared for demonstration of salivary peroxidase (SP) activity using DAB as chromogen and no heavy metal counterstains in a previous study. Endogenous substrates also promoted cytochrome C oxidase (CCO) activity in the mitochondria. (A) Terminal bud at age one day after birth, in which some cells show SP activity in the rough endoplasmic reticulum (RER, arrowheads), but very little in the newly forming secretory granules (g). CCO activity (roughly proportional to the reaction product density in the cristae) varies considerably among the scattered, mostly small mitochondria (arrows). (B) At age 42 days, secretory granules and RER show strong SP activity in the acini (a) but not the intercalated duct (i). Mitochondria are modest in size and number, and vary considerably in CCO activity (arrows) in the acini, intercalated ducts, and myoepithelial cell processes (arrowheads). A $\times 6700$; B, $\times 6200$. This figure is composed of portions of previously published illustrations (Redman and Field 1993, *Anat. Rec.* 235: 611–621).

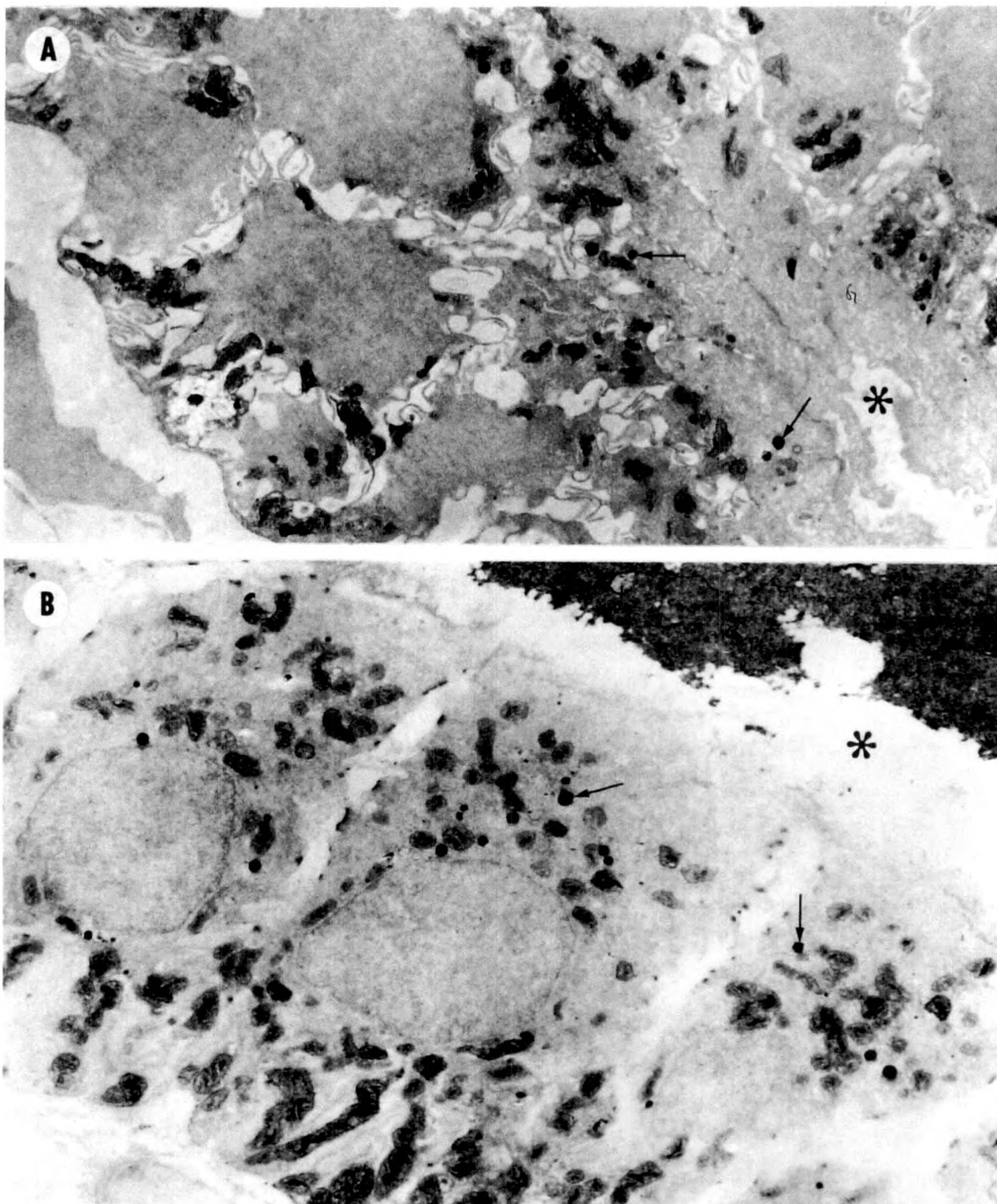


Fig. 7. In developing striated ducts at (A) 7 and (B) 28 days, the mitochondria are longer and more numerous with age, and almost all show strong CCO activity. Peroxisomes (arrows) also are seen in the luminal cytoplasm. The secretory product (homogeneous dark mass in the upper right) in the lumen (*) of the duct in (B) shows moderate SP activity. Both $\times 6400$.

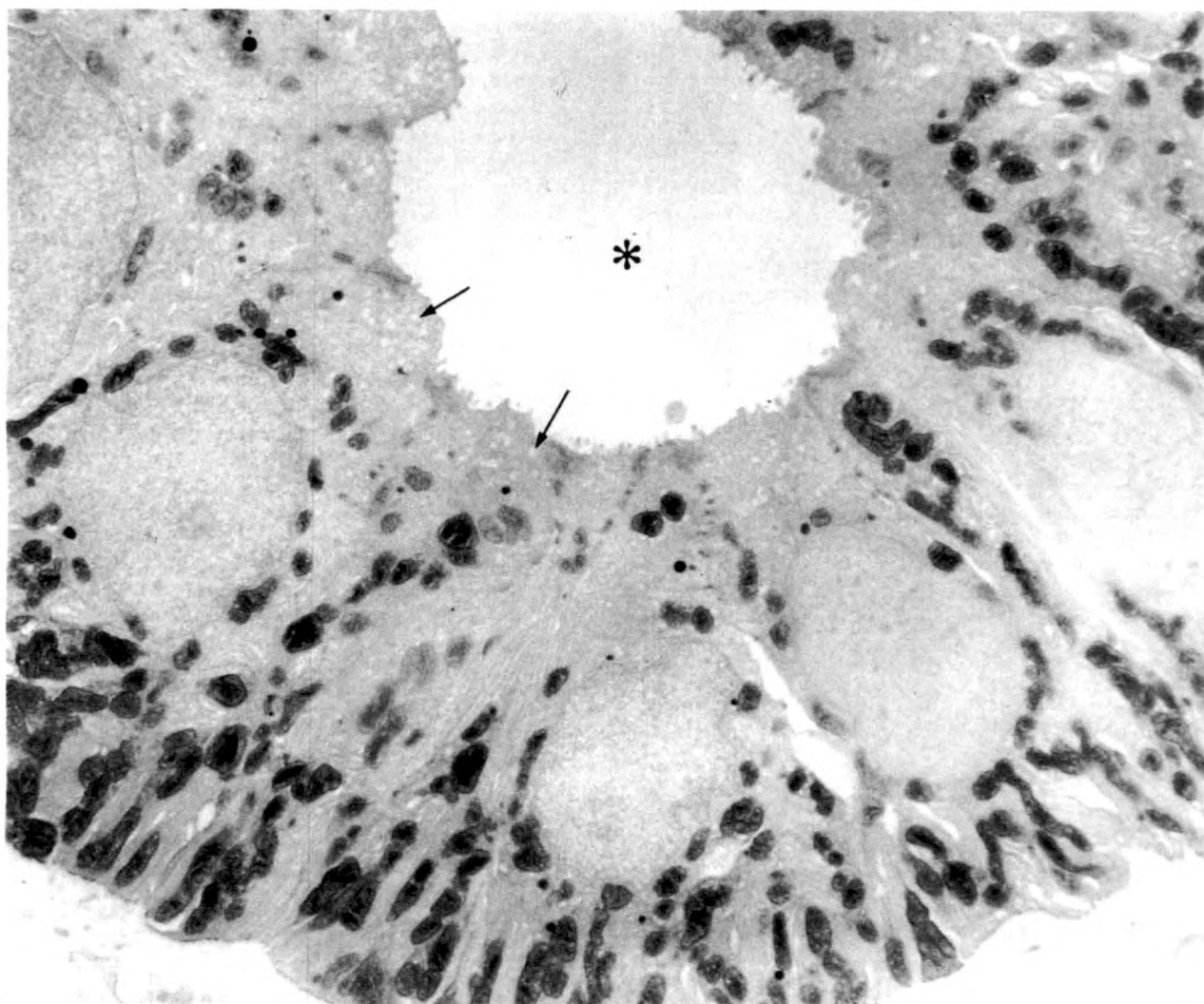


Fig. 8. In this striated duct at age 42 days, almost all of the mitochondria are very long, show strong CCO activity, and reside in the basal cytoplasm. Many small vesicles (arrows) are present in the cytoplasm subjacent to the lumen (*). $\times 6200$.

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A new stain for identification of avian leukocytes

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Abstract

Differential staining of avian leukocytes was achieved within 6 min following brief fixation in a methanolic solution of C.I. acid red 360 followed by immersion in a mixture containing C.I. basic blue 41, C.I. basic blue 141, and C.I. acid red 52. Heterophils contained black angular and punctate granules. Eosinophils contained bright purple granules. Lymphocytes displayed red nuclei and blue cytoplasm. Monocytes contained red-brown nuclei and lavender cytoplasm. Basophils showed red-orange granules. Thrombocytes stained deep purple. Compared to traditional panoptic stains like Wright's or Giemsa's, the new staining method provides brighter colors, more precise details of cellular structures, and shorter staining time. Significantly, it facilitates identification of avian leukocyte species based on differences in color as well as differences in size and shape.

Key words: acid anionic dye, avian leukocytes, basic cationic dye, basophils, blood smear, eosinophils, heterophils, lymphocytes, monocytes

In avian hematology, identification of different kinds of leukocytes contained in a blood sample has been both challenging and controversial. Challenges have arisen because of the quest for a stain that would represent an improvement over conventional panoptic stains like Wrights or Giemsa. Controversies have arisen because with the conventional stains there is no universal agreement on the staining characteristics of most types of avian leukocytes (Campbell 1995, Lucus and Jamroz 1961). For example, differential staining of avian eosinophils and heterophils is at best imperfect, because granules in both types of cells stain orange owing to their affinity for the eosin in panoptic staining mixtures. Practically speaking, one can tentatively identify a heterophil based on orange staining angular granules. Similarly, one can presumptively identify an eosinophil based on orange staining round or globular granules.

Although both heterophil and eosinophil granules stain orange, Campbell (1995) proposed that the orange color is brighter in eosinophil granules than in heterophil granules. Compounding the problem is the fact that heterophil granules can sometimes be round rather than oval or angular, and eosinophil granules can be oval or angular rather than round (Campbell 1995, Lucus and Jamroz 1961).

For accuracy, most would prefer to distinguish heterophils from eosinophils based on differences in the color of their granules rather than differences in their shapes and degree of brightness of the same color. Similarly, distinctions between avian monocytes and large lymphocytes using panoptic stains have been made largely on the basis of size rather than differences in color.

For those analyzing samples of avian blood, it is obvious that there are shortcomings in the ability of routine panoptic stains to distinguish reliably the various major types of avian leukocytes. For example, dipping stains may be fast, but they do not stain all avian leukocytes differentially. Also, their staining reactions are often pale. Although fluorescent stains like fluorescein isothiocyanate (Rath et al. 1998) and saffron (Trigoso and Sockert

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1995) have been evaluated as stains for avian blood, they identify heterophils, but do not stain heterophils and eosinophils differently.

This report describes a new staining method for differential coloration of leukocytes in samples of avian blood. The method permits distinction of one leukocyte species from another based on differences in color as well as in size and shape.

Materials and methods

Reagents

Acid dye A

Make a 1% methanolic solution of C.I. acid red 360 (Telon Fast Red AFG, Mobay Chemical Co., Pittsburgh, PA) by dissolving 1 g powdered dye in 100 ml absolute methanol (methanol, ACS grade, Fisher Scientific, Pittsburgh, PA) in a plastic bottle with screw cap. Agitate to dissolve, and keep tightly capped at room temperature. At room temperature, the staining properties of the solution are stable for one year. Label as Acid Dye A.

Staining mixture B

Make separate stock solutions of each of the following dyes:

1. One percent phosphate buffered aqueous solution of C.I. basic blue 41 (Remacryl blue BRL, Hoechst, Charlotte, NC) prepared by adding deionized water up to 1 l to 8.9 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ and 4.6 g KH_2PO_4 (Fisher Scientific, Pittsburgh, PA) in a volumetric flask to make a buffer solution at pH 6.8. Add 1 ml liquid phenol (Fisher Scientific) as a preservative and store in a plastic bottle with screw cap. At room temperature, the solution is stable up to one year. Add 100 ml of this buffer solution to 1 g of the powdered basic blue 41 dye contained in a plastic bottle. Agitate to dissolve and store in the same plastic bottle with screw cap. At room temperature, the staining properties of the solution are stable for one year. Label as BB 41.
2. One percent aqueous solution of C.I. basic blue 141 (Basacryl blue X7GFL, BASF, Leverkusen, FRG) prepared by adding 100 ml deionized water to 1 g of the powdered dye contained in a plastic bottle. Agitate to dissolve and store in the same plastic bottle with screw cap. At room temperature, the staining properties of the solution are stable for one year. Label as BB 141.
3. One percent aqueous solution of C.I. acid red 52

(Intracid rhodamine B, Crompton and Knowles, Charlotte, NC) prepared by adding 100 ml deionized water to 1 g of the powdered dye contained in a plastic bottle. Agitate to dissolve and store in the same plastic bottle with a screw cap. At room temperature, the staining properties of the solution are stable for one year. Label as AR 52.

Rinse buffer C

1. Aqueous phosphate buffer solution, pH 5.6, prepared by adding deionized water up to 1 l to 0.7 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ and 8.7 g KH_2PO_4 in a volumetric flask to make a pH 5.6 buffer solution. Agitate thoroughly until buffer salts are completely dissolved, add 1 ml liquid phenol, and store in a plastic bottle with screw cap. At room temperature, the solution is stable for one year. Label as Rinse buffer C.

To prepare the staining mixture, designated as Staining mixture B, put 5 ml stock solution BB 41, 5 ml stock solution BB 141, and 5 ml stock solution AR 52 into a small plastic bottle. Agitate to mix, filter using medium porosity filter paper, and store as "Staining mixture B" in a separate bottle with a screw cap. At room temperature, the staining properties of Staining mixture B are stable for one year.

Staining procedure

1. Make coverslip smears from avian blood samples anticoagulated with EDTA and air dry. Do not fix the smears. In the present study, blood samples were obtained from a variety of avian species including the orders psittaciformes (parrots), falconiformes (falcons, eagles, vultures, hawks), galliformes (chickens), strigiformes (owls), anseriformes (ducks), and piciformes (barbets, toucans, woodpeckers).
2. Immerse unfixed smears on coverslips in a small covered Coplin staining jar containing Acid dye A.
3. After 1 min, remove the coverslip with a forceps, and without washing, place immediately into a small covered Coplin jar containing Staining mixture B.
4. After 5 min, remove the coverslip with a forceps and rinse by vigorous agitation in a beaker containing Rinse buffer C for 10 sec.
5. Blot gently on filter paper, air dry, and mount face down on a glass slide with a drop of liquid cyanoacrylate adhesive (Super Glue, Loctite Corp., Cleveland, OH). View under the light

microscope. Using a didymium glass filter over the light source of the microscope, red and orange colors are intensified; this improves discrimination of different types of leukocytes based on color differences.

Spectral analysis

UV and visible spectral curves were obtained using a Hewlett Packard diode array spectrophotometer (Hewlett Packard, Palo Alto, CA) for dilute aqueous solutions of C.I. acid red 360, C.I. basic blue 41, C.I. basic blue 141, and C.I. acid red 52. UV and visible spectral curves were also obtained for the mixture of basic blue 41, basic blue 141, and acid red 52. Analysis of the mixture was performed to ascertain whether it was composed of three individual dyestuffs with spectral curves typical of each dye alone, or whether new compounds were formed as the result of different dyes reacting in the mixture.

Two different lots of each of the dyestuffs used in the staining method were also evaluated.

Results

Stained in the manner described, nucleated erythrocytes had bluish orange cytoplasm and red-brown nuclei. Some erythrocytes showed purple cytoplasm, and may represent immature erythrocytes or reticulocytes.

Leukocytes displayed a variety of colors as shown in Figure 1, row 1. Heterophils contained many thin crescentic or splinter-shaped granules that stained black. In some species, punctate black stained granules occurred, and nuclei appeared red.

Eosinophils contained bright purple round or globular shaped granules, and red nuclei (Fig. 1, row 2). In some species, granules in eosinophils were numerous, and appeared to be compressed and flattened. Conceivably, these granules might actually have been round, but appeared flattened because they were so numerous and tightly packed together. Based on color, granules in eosinophils could be distinguished easily from granules in heterophils as depicted in Figure 1, row 3.

Basophils displayed red granules and red-brown nuclei as seen in Figure 1, row 3. Thrombocytes were dark purple and often contained one or more clear vacuoles. These darkly stained thrombocytes could be distinguished easily from lymphocytes, which had red nuclei and blue cytoplasm.

In Figure 1, row 4, lymphocytes showed blue cytoplasm and red nuclei. Occasionally, lymphocytes contained a few orange cytoplasmic granules. Mononuclear cells that might be regarded as

atypical lymphocytes had abundant deep blue cytoplasm and red-brown nuclei.

As shown in Figure 1, row 4, monocytes had lavender colored cytoplasm and red-brown, bean-shaped nuclei. Frequently, clear vacuoles of varying size and shape appeared in the cytoplasm of monocytes. Some monocytes contained cytoplasmic pseudopodia and dust-like red or pink granules.

The spectra of individual dyestuffs are displayed in Figure 2. Basic blue 41 showed a peak at 608 nm. Basic blue 141 had a major peak at 636 nm and a minor peak at 256 nm. Acid red 52 had a major peak at 564 nm, and small peaks at 524, 352, and 256 nm. Basic red 360 showed a major peak at 534 nm and minor peak at 292 nm. The spectrum of the mixture of basic blue 41, basic blue 141, and acid red 52 is shown in Figure 3. There was a major peak at 628 nm and minor peaks at 260 and 300 nm. The major peak in the mixture did not correspond to the major peaks of any of the three dyestuffs in the mixture, suggesting the formation of new compounds.

Different lots of the dyestuffs used in the staining procedure yielded identical results for avian blood smears. The staining properties of a mixture of basic blue 41, basic blue 141, and acid red 52 were undiminished after storage at room temperature for one year. Staining reactions on blood smears between 6–12 months old and stored at room temperature were identical to the reactions obtained on freshly prepared slides.

Discussion

Dyestuffs used in the present study have been used individually as stains for human leukocytes (Kass 1987a,b,c, 1988, 1992, 1993). An extensive computer-based literature search revealed that they have not been used in an aqueous mixture containing two basic cationic dyes and one acid anionic dye for staining avian leukocytes.

The new staining method described here involves three steps that do not require repetitive dipping. The first step is brief immersion in a methanolic solution of an acid anionic dye. The second step is immersion in a mixture containing two basic cationic dyes and one acid anionic dye. The third step is a brief vigorous rinse in a phosphate buffer.

To the best of our knowledge, the staining procedure is the first contemporary stain for avian leukocyte identification that incorporates a methanolic solution of an acid anionic dye as the first step in the procedure. It may also be the first staining

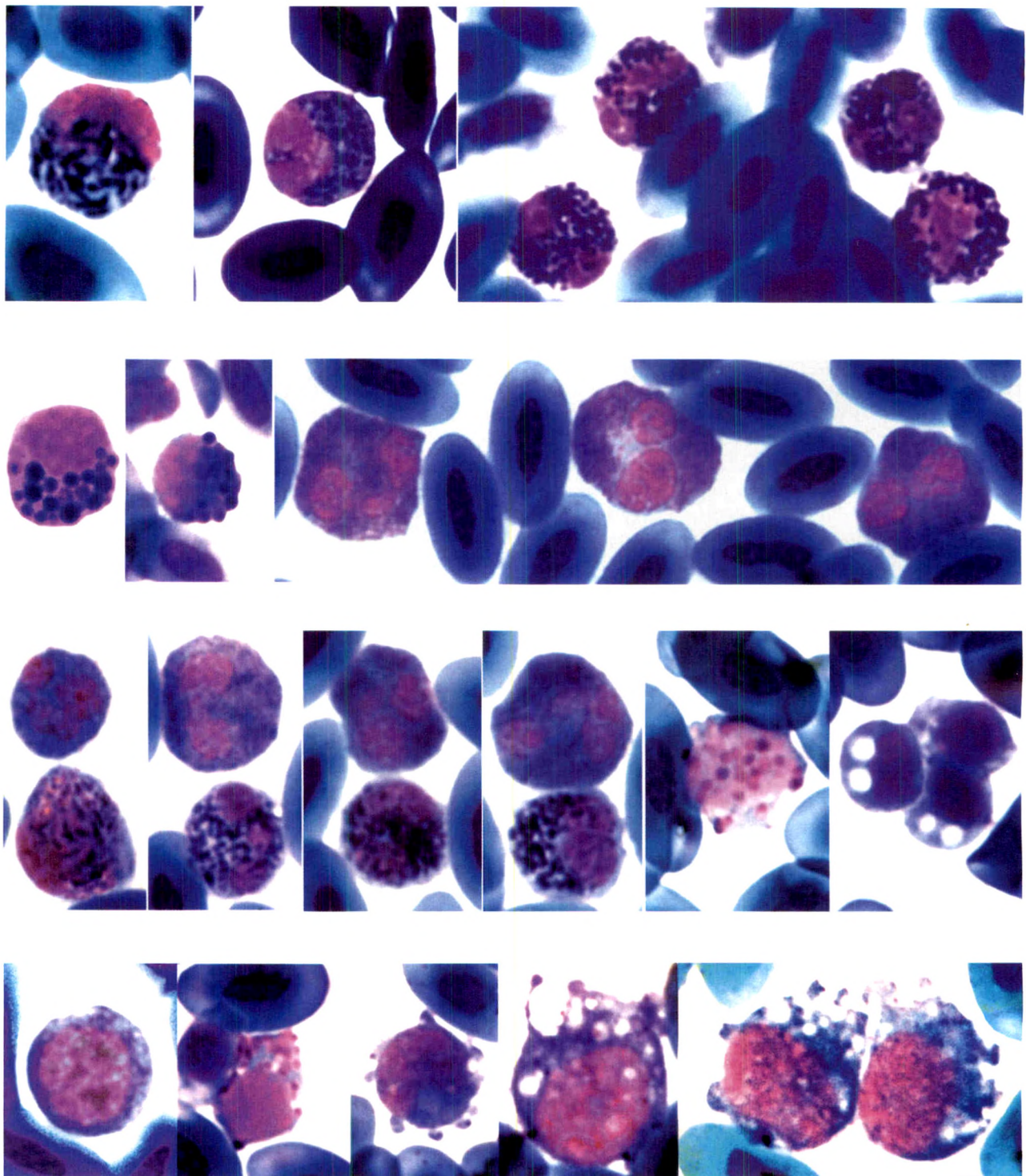


Fig. 1. Row 1, left to right: heterophil with black-stained granules (galliforme), heterophil with prominent black punctate granules (psittaciforme), four heterophils in a bird with heterophilia (psittaciforme). Row 2, left to right: eosinophil with purple globular granules (piciforme), eosinophil with purple globular granules (psittaciforme), eosinophilia showing eosinophils with numerous flattened granules that stain purple (anseriforme). Row 3, left to right: four examples of a heterophil next to an eosinophil (anseriforme), basophil with red granules (psittaciforme), thrombocytes (galliforme). Row 4, left to right: lymphocyte (psittaciforme), lymphocyte containing orange-stained granules (psittaciforme), small lymphocyte with pseudopodia (galliforme), monocyte with prominent pseudopodia and violaceous cytoplasm (psittaciforme), monocytes with prominent pseudopodia and violaceous cytoplasm (psittaciforme). Original magnification $\times 1000$.

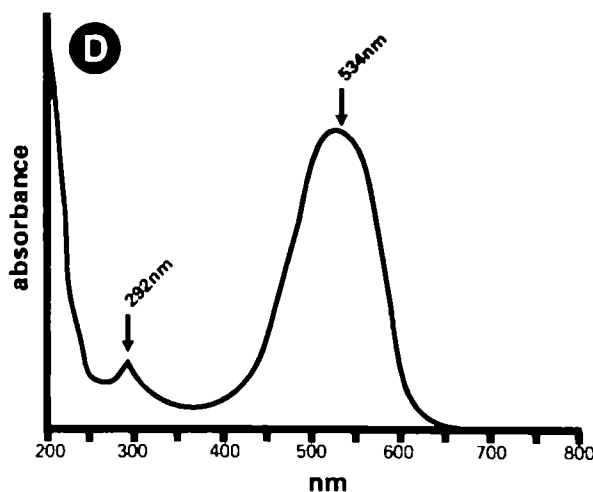
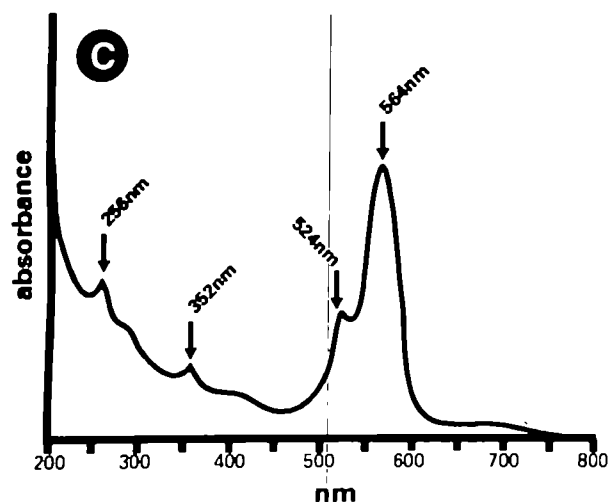
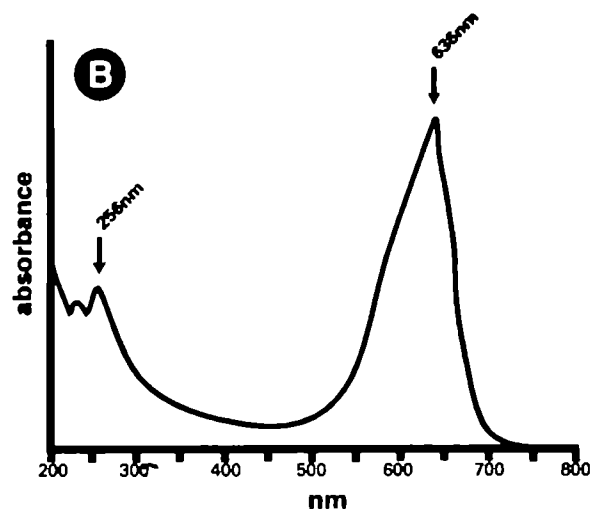
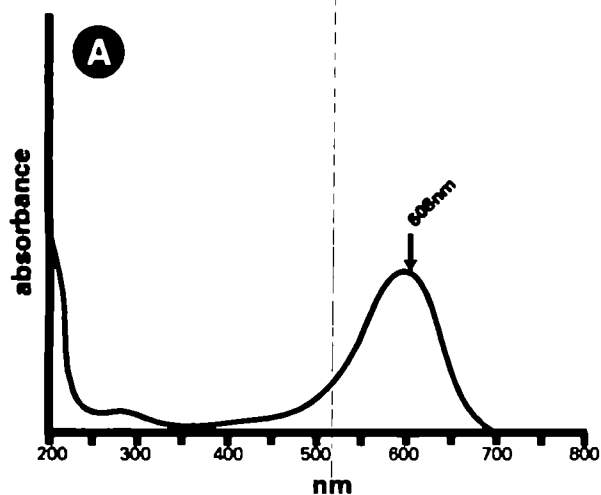


Fig. 2. Spectral curves of individual dyestuffs used in the avian leukocyte stain. A) Basic blue 41. B) Basic blue 141. C) Acid red 52. D) Acid red 360.

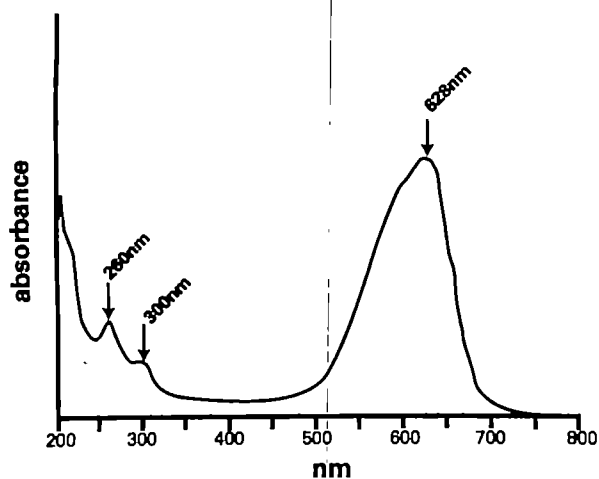


Fig. 3. Spectral curve of a mixture of basic blue 41, basic blue 141, and acid red 52.

mixture for avian leukocytes that differentially stains the granules of heterophils and eosinophils using ordinary incandescent light microscopy. As yet, mechanisms responsible for the staining reactions are unknown, and substances within the cells that react with the components of the stain to form characteristic colors have not been identified.

As seen from the spectral curves, the peak absorbance in the staining mixture differs from peak absorbances of the individual dyes that constitute the mixture. This finding suggests the formation of new compounds in the mixture. Further studies will be required to identify and characterize the species responsible for the peaks in the mixture.

Once the stable stock solutions of dyes, staining mixture, and rinse buffer are prepared, the staining method itself involves only transfer of a coverslip from one Coplin staining jar to the next in sequence,

with a brief buffer rinse at the conclusion of the staining reaction; the total time required from start to finish is 6 min. For comparison, a traditional Giemsa stain as described in the literature takes at least 30 min to perform (Miale 1982), and Wright's staining requires 8–10 min (Williams 1990).

As improvements in the identification of avian leukocytes occur, we expect to gain greater understanding of the significance of changes in the composition of these cells in health and disease. For example, we might be better able to identify changes in the staining characteristics of leukocytes as a reflection of their exposure to microorganisms and various environmental toxins. Further studies will be required to evaluate the ability of the staining procedure presented here to identify immature granulocytes and developing erythroblasts in avian bone marrow specimens.

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Kinetic studies of glutaraldehyde binding in liver

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Abstract

To study the kinetics of glutaraldehyde fixation, fresh rabbit liver cubes were immersed in 3% buffered ³H-glutaraldehyde for various periods of time. Following weighing and a brief rinse in water, the tissues were solubilized, and the radioactivity was measured in a scintillation counter. Binding of the isotope was half-maximal after approximately 4 h and a plateau was reached after approximately 20 h. We also investigated the reversibility of glutaraldehyde fixation. Fixed liver cubes were weighed and immersed in water for various periods of time, and after solubilization, the radioactivity was determined. After rinsing for 48 h, approximately 95% of the radioactivity was lost from the tissue specimens, indicating that fixation with glutaraldehyde is largely reversible. Light and electron microscopy of specimens rinsed for 1 and 48 h showed essentially similar morphology. Rinsing for 48 h restored some of the immunoreactivity that was absent after rinsing for only 1 h.

Key words: electron microscopy, fixation, glutaraldehyde, immunohistochemistry, kinetics, light microscopy, liver

Glutaraldehyde was introduced as a fixative for electron microscopy by Sabatini et al. in 1962, and has since enjoyed great popularity. Glutaraldehyde acts mainly by cross-linking proteins (Hayat 1981). There has been considerable discussion concerning the best conditions for fixation with glutaraldehyde (Hayat 1981) including the duration of fixation. While structural preservation is often excellent for light and electron microscopy, the loss of antigenic properties in the fixed tissues, particularly after fixation by vascular perfusion, has reduced the usefulness of glutaraldehyde as a fixative for immunohistochemistry. Several methods have been designed to restore the antigenicity of fixed tissue proteins including digestion with proteinase K or trypsin, or heating, but these methods frequently result in loss of structural details.

The aim of the study reported here was to investigate the kinetics of glutaraldehyde binding to tissues under conditions similar to those in a surgical pathology laboratory. In addition, we tested the hypothesis that glutaraldehyde binding is a reversible process.

Materials and methods

Binding of glutaraldehyde

Forty tissue specimens were cut from a fresh rabbit liver. The specimens were roughly cuboidal, a side measuring 4–6 mm. The specimens were put into a vial containing 100 ml 3% glutaraldehyde (Biological Grade, Ted Pella, Redding, CA) in 0.1 M phosphate buffer, pH 7.2. The fixative also contained 100 µCi of ³H-glutaraldehyde (specific activity 5 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO). Two specimens were removed, at each time point after fixation: 15 min, 30 min and 1, 2, 4, 20, 47, 96, 166 and 214 h. Each specimen was then put into 10 ml of distilled water for 1 h to remove most of the unbound glutaraldehyde. They were then blotted on a filter paper,

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weighed, and put into a scintillation vial containing 1 ml of Solvable™ (Packard, Meriden, CT) for solubilization. For comparison, two specimens were taken after 15 min fixation and weighed without the preceding 1 h rinsing period. The dissolution of the tissues took several days and was aided by increasing the temperature to 40° C. Ten milliliters of scintillation fluid (Hiionic Fluor, Packard, Meriden, CT) was then added to 0.1 ml of the dissolved tissue, and the tritium content was determined using an LKB 1215 Rackbeta II liquid scintillation counter (Wallac, Gaithersburg, MD). Samples taken from the fixative before adding the tissue specimens and at the end of the fixation period (214 h) showed that the loss of radioactivity averaged about 9% compared to unused fixative.

Reversibility of glutaraldehyde binding

Another 40 tissue cubes were put into another vial with 40 ml fixative of the same composition as above. After 22 h, the specimens were removed, rinsed briefly in distilled water, and put into a second vial containing 50 ml water. Two of these specimens were removed immediately, blotted with filter paper and weighed. Additional specimens were removed after 1, 22, 48, 72, 168, 216 and 312 h in distilled water. At each time point, two specimens were removed, blotted and weighed.

Each tissue specimen was then put into a scintillation vial and 1 ml of Solvable™ was added. When the tissue was dissolved, 10 ml of Hiionic-Fluor was added to 0.1 ml of the dissolved tissue and the radioactivity measured.

To study the effects of rinsing on the structure of the tissue, other tissue cubes were fixed for 22 h in nonradioactive 3% glutaraldehyde as above. They were rinsed for either 1 or 48 h in distilled water, dehydrated for 30 min in 70% ethanol, 30 min in 95% ethanol and three 30 min baths in 100% ethanol, passed through two 30 min baths of xylene, and embedded in paraffin; 4 µm thick sections were picked up on glass slides and stained with hematoxylin and eosin.

Additional paraffin sections were processed for detection of immunoreactivity that might remain after glutaraldehyde fixation and rinsing. For this purpose we used antibodies against fibronectin, a protein that is abundantly present in liver (Kim et al. 1997). After removal of paraffin and rehydration, the sections were treated with 1.5% H₂O₂ in distilled water for 15 min to block endogenous peroxidase activity. They were then kept in 2% bovine serum albumin (BSA) for 30 min, followed by exposure to antibodies against rat fibronectin

raised in goat (ICN/Cappel, Costa Mesa, CA) for 30–40 min. The antibody was diluted 1:50, 1:100, 1:200 and 1:300 with 1% BSA. Control sections were exposed to phosphate buffered saline (PBS) or 2% goat serum in PBS instead of the primary antibody. After rinsing in PBS, horseradish peroxidase coupled donkey anti-goat serum (ICN/Cappel) diluted 1:500 was applied for 30 min. Finally, diaminobenzidine was used for visualization of the peroxidase.

To detect ultrastructural consequences of the extended rinsing, electron microscopy studies were carried out on approximately 1 mm³ tissue pieces removed from the periphery of the liver cubes fixed as above for 22 h, and subsequently rinsed for 1 or 48 h. The cubes were post-osmicated, dehydrated, and embedded in epoxy resin according to standard procedures. For a light microscopy control, 1 µm sections were stained with toluidine blue, pH 7. Ultrathin sections were contrasted with uranyl acetate and lead citrate.

All procedures, except the dissolution of the tissues, were carried out at room temperature and with no stirring.

Results

The binding of ³H-glutaraldehyde increased up to approximately 20 h of fixation (Fig. 1). Half-maximal binding was reached after approximately 4 h.

The isotope concentration in the tissues declined

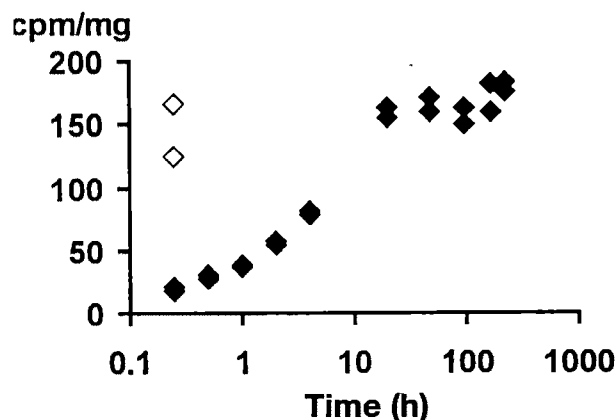


Fig. 1. Semilogarithmic diagram showing the radioactivity of rabbit liver cubes (two specimens for each time point) fixed in ³H-glutaraldehyde for various periods of time. Saturation with isotope is reached after approximately 20 h fixation, with half-maximal binding after approximately 4 h. Open symbols show the radioactivity in two specimens that were fixed for 15 min, but not rinsed.

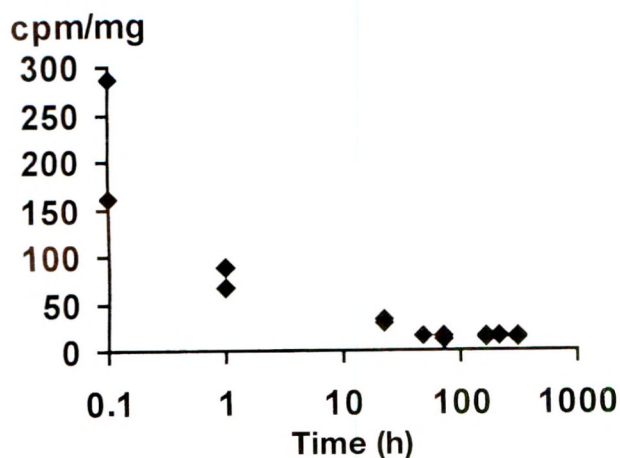


Fig. 2. Semilogarithmic diagram showing the radioactivity remaining in rabbit liver cubes fixed for 22 h and subsequently rinsed in distilled water for various periods of time. After 1 h, ~50% of the radioactivity remained. Rinsing for 48 h or more reduced the radioactivity by approximately 95%.

The isotope concentration in the tissues declined rapidly, reaching 50% of the initial level within 1 h (Fig. 2). After rinsing for 48 h, isotope concentration in the tissues was approximately 5% of the initial concentration.

Light microscopy revealed no significant differences between tissues rinsed for 1 and 48 h in either the hematoxylin and eosin stained sections (Fig. 3a, b) or toluidine blue stained epoxy sections.

Immunohistochemical staining for fibronectin showed reaction in the perisinusoidal space as described earlier (Kim et al. 1997). Immunoreactivity was moderate, but significantly stronger in specimens rinsed for 48 h than in those rinsed for 1 h (Fig. 4a,b). Immunoreactivity was generally stronger in the periphery of the tissue cubes than in the center. Antibody diluted 1:50 gave the most clear-cut results.

Under the electron microscope, tissues rinsed for 48 h showed slightly less contrast than those rinsed for 1 h (Fig. 5), but the structure of the tissues was well preserved. The outer mitochondrial membranes were sometimes lost from tissues rinsed for 48 h.

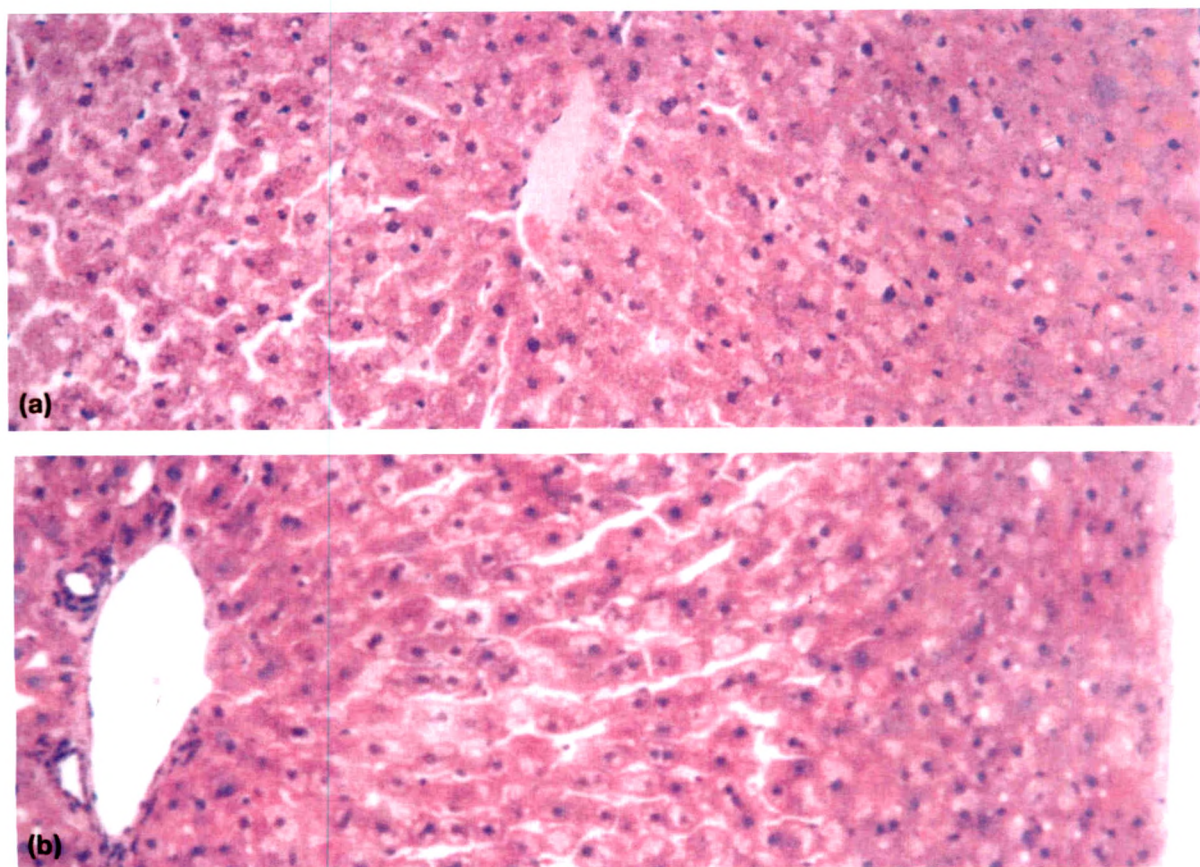


Fig. 3. Light micrographs of rabbit liver fixed for 22 h in glutaraldehyde: A) rinsed for 1 h, and B) rinsed for 48 h. There were no significant differences in the structure. Hematoxylin and eosin. $\times 150$.

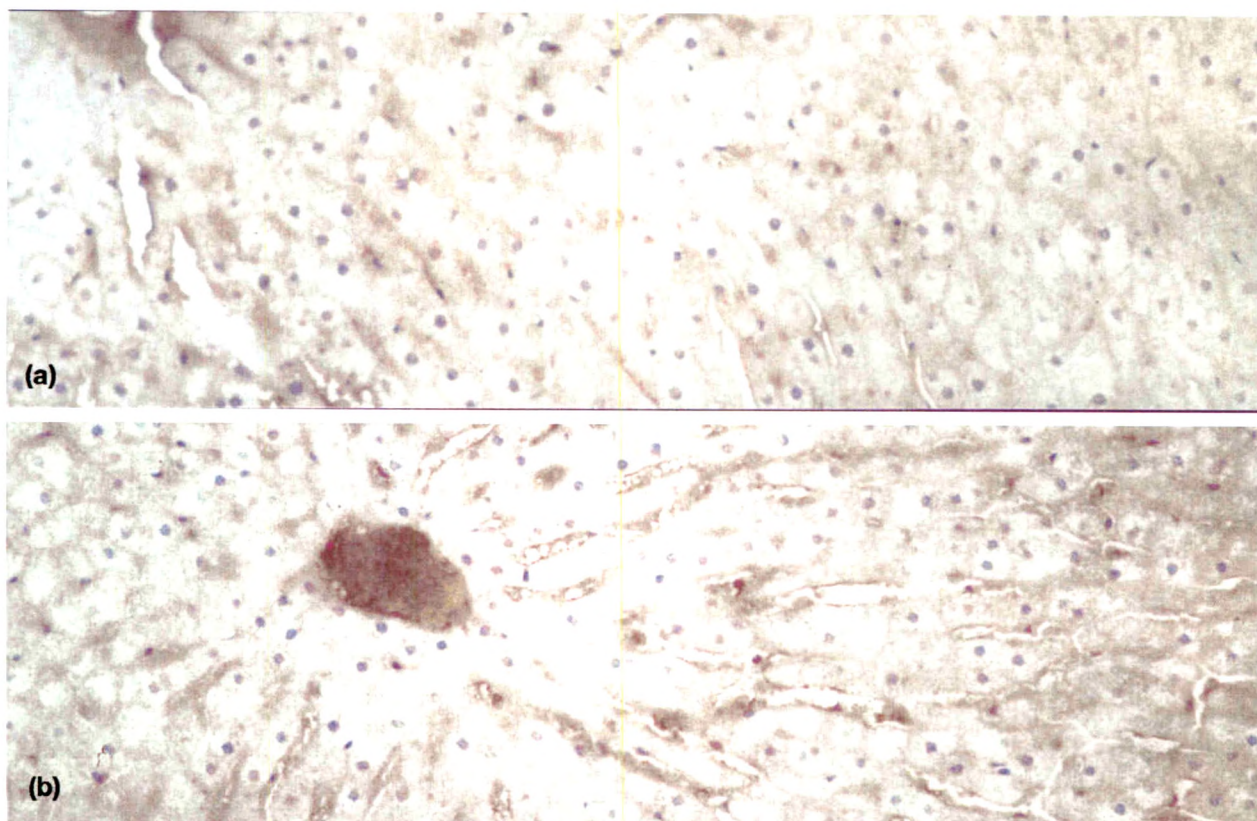


Fig. 4. Rabbit liver fixed for 22 h in glutaraldehyde: A) rinsed for 1 h, and B) rinsed for 48 h, immunostained with antibodies against fibronectin (primary antibody dilution 1:50). Immunoreactivity was observed mainly in the perisinusoidal space and was slightly greater in the specimens rinsed for 48 h. $\times 300$.

Discussion

Ericsson and Biberfeld (1967) investigated the penetration of and fixation by glutaraldehyde in rat liver specimens. They observed a yellowish brown zone in the outer region of the specimen where tissue preservation was good, as judged by light and electron microscopy. In subjacent layers, the tissue was more whitish and the preservation of structure was poor. The thickness of the outer zone reached 1 mm after 40 min fixation and was found to be proportional to the square root of the duration of fixation. Those findings confirmed Medawar's (1941) earlier observation that fixatives obey Fick's diffusion law.

McDowell and Trump (1976) used 2 cm cubes of human liver after 1, 4 and 24 h fixation. After 24 h fixation, penetration of the fixative, judged by a color change of the tissue, averaged 6 mm. Good fixation was seen in a peripheral 3.5 mm-wide zone, whereas only the outermost 1.8 mm of tissue reacted positively to Schiff's reagent, without prior oxidation with periodic acid, indicating the presence of aldehyde groups.

Fixation can be divided into two phases: diffusion of the fixative into the tissue and the actual binding of the fixative to tissue components (e.g., cross-linking proteins). Both Ericsson and Biberfeld (1967) and Hopwood (1967) found that the diffusion rate for glutaraldehyde was slow compared to that of formaldehyde. In contrast, glutaraldehyde binding was faster.

In the present study, the rate of glutaraldehyde binding was measured using radioisotopes. Binding of ^3H -glutaraldehyde in 4–6 mm liver cubes reached its half-maximal level after 4 h of immersion, and saturation after approximately 20 h. A similar study of ^{14}C -formaldehyde binding kinetics (Helander 1994) demonstrated half-maximal binding levels after approximately 100 min, and saturation after approximately 24 h, confirming that formaldehyde is a faster fixative than glutaraldehyde.

It has been stated previously that glutaraldehyde, unlike formaldehyde, can be removed only partly from the fixed tissues (Høyer et al. 1997). In the present study, the reversibility of ^3H -glutaraldehyde binding is clearly demonstrated; only a small fraction ($\sim 5\%$) remained after 48 h of rinsing

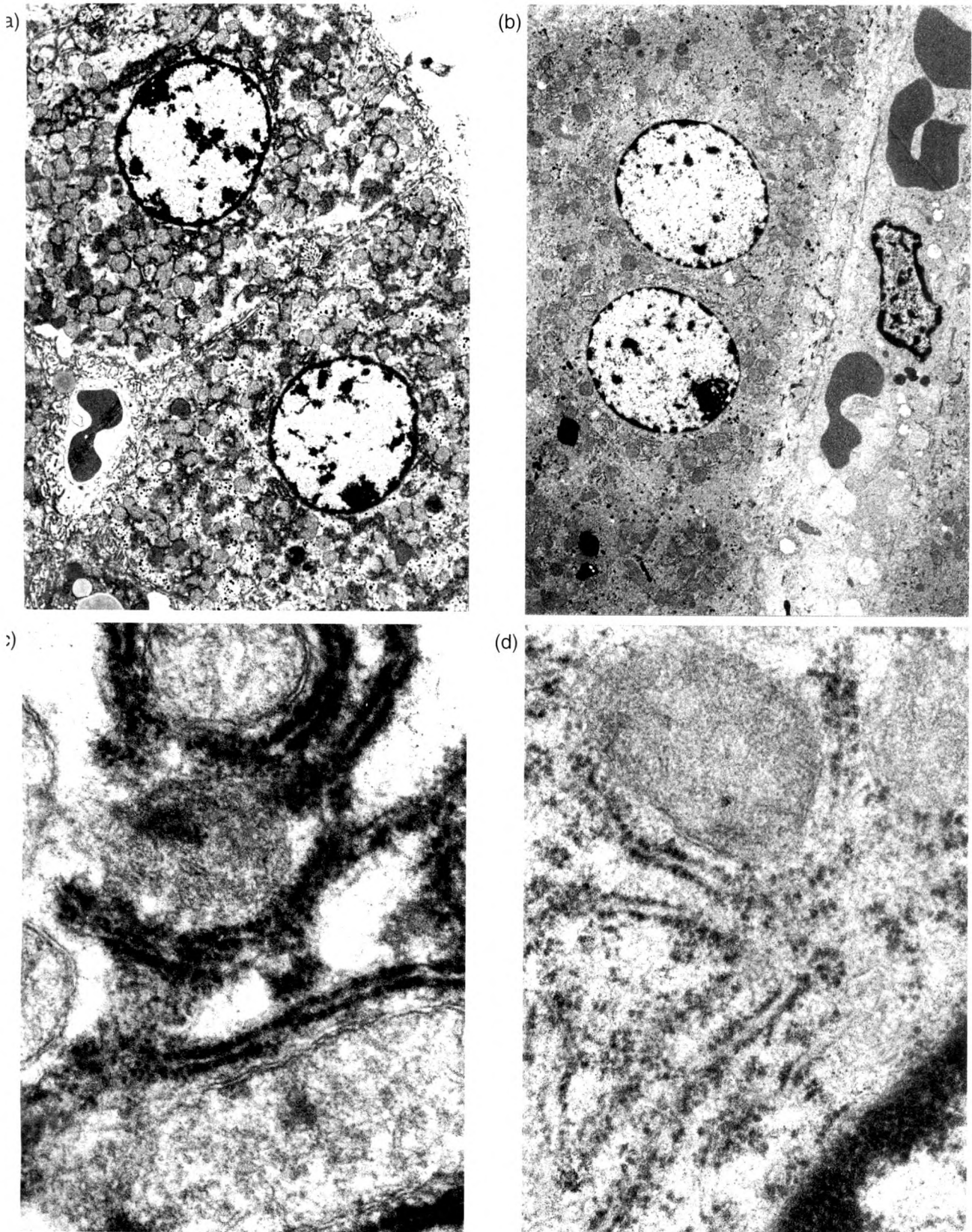


Fig. 5. Electron micrographs of rabbit liver fixed for 22 h in glutaraldehyde: A) and C) rinsed for 1 h, and B) and D) rinsed for 48 h. At low magnification (A and C; $\times 1300$) there were no significant differences in ultrastructure, but the contrast was higher in specimens rinsed for 1 h than in those rinsed for 48 h. The same differences in contrast were observed also at high magnification (B and D, $\times 72\,000$), while the ultrastructure was similar.

in water. Previous studies have demonstrated similar reversibility of formaldehyde fixation of rabbit liver (Helander 1994). The loss of glutaraldehyde isotope from the tissue blocks presumably reflects reversibility of the binding of glutaraldehyde to the proteins. This would also explain the apparent return of some immunoreactivity. One cannot rule out, however, that loss of glutaraldehyde-protein complexes contributes to the loss of isotope, although we presume that this accounts for a small proportion of loss of radioactivity.

Removal of the glutaraldehyde by rinsing for 48 h did not significantly affect the light microscopic structure of the tissues. By electron microscopy, a small loss of contrast was noted, but no other significant differences were observed. Immunohistochemical staining with antibodies against fibronectin was positive. Further studies are required in other tissues, with other antibodies, and with other rinsing periods.

Acknowledgments

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Validation of image analysis for enzyme histochemical and immunocytochemical staining

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Abstract

Immunocytochemical and enzyme histochemical analyses of cells and tissues are used to detect changes in the extent of injury and the expression of various molecules. Image analysis quantitation offers an easier, more efficient technique to evaluate these changes. We studied the application of image analysis for evaluating enzyme histochemistry and immunocytochemistry of cells and tissues as a way to assess stroke. Using brain sections, we compared investigator and computer-generated image analysis of 2,3,5-triphenyltetrazolium chloride stained cerebral infarcts in rats subjected to 2 h middle cerebral artery occlusion and 22 h re-perfusion. Both methods documented the infarct volumes with a comparison of means of less than 5%. This suggests no difference between computer- and hand-calculated values. Computer-generated analysis was easier and faster to use. Using endothelial cell monolayers, immunocytochemical staining of a time course of heat shock protein expression was compared to a grading system using fast red chromagen counterstained with hematoxylin. Results demonstrated greater ease and efficiency with computer-generated image analysis compared to other subjective systems of analysis. Image analysis is more useful for detecting small differences in staining, especially when using 3,3'-diaminobenzidine as a chromagen. Investigator bias is also reduced using this system. Our comparisons validate the use of this versatile technology to assess more easily both cell and tissues in stroke research.

Key words: enzyme histochemistry, image analysis, immunocytochemistry

Innovations in CCD camera technology and the availability of sophisticated software allow researchers to analyze complex images quickly (Grant 2001). The ease of use and flexibility of image analysis make it an important tool for the research scientist. Although image analysis software and system applications have been available for decades, its use has been limited by the complexity of

the systems. We present here the application of this technology for evaluating and analyzing cells and tissues using a single software program.

Computerized image analysis offers several important advantages that make it a useful and versatile analytical tool. Specific features of interest in an image can be extracted by digitizing the image and using processing routines such as image subtraction and contrast enhancement (Berger et al. 1999). Lamaziere et al. (1993) showed that image analysis facilitates quantitative analysis and allows more effective comparisons of different immunohistochemical staining densities. Computer image analysis has also been shown to reduce investigator bias and is more time efficient and cost effective, especially when studying large numbers of tissue sections (Kohlberger et al. 1999, Bovik et al. 1992).

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The middle cerebral artery occlusion model (MCAO) of focal neuronal ischemia has become an important research tool for three reasons. First, the model most closely approximates the actual events that occur during occlusion of a pre-cerebral vessel. Second, compared to global ischemia models, volumetric analysis of stroke size is facilitated owing to the ability to compare with the unaffected cerebral hemisphere. Third, the changes can be evaluated at the cellular level.

Image analysis has proven useful for this stroke model because rapid and accurate detection of infarct location and size is important for assessing the efficacy and potency of therapeutic interventions (Calloway et al. 2000). Image analysis programs available currently can be used to analyze cerebral infarct samples using both enzyme histochemical and immunohistochemical staining methods (Champelovier et al. 1991, Kohlberger et al. 1999).

Enzyme histochemistry using indicators such as 2,3,5-triphenyltetrazolium chloride (TTC) analyzed by computerized image analysis software has been shown to be a useful tool to measure the size of infarcts in rat brains (Goldlust et al. 1996, Vinall et al. 2000).

Immunocytochemistry has been used extensively to detect a variety of proteins expressed both during and after ischemia. Heat shock protein 72 (hsp72), a member of a family of molecular chaperones, is one of these proteins (Li et al. 1992, Longo et al. 1993, Kato et al. 1991). More importantly, hsp72 is produced in varying degrees by ischemic neurons, particularly in populations of cells recovering from the ischemic injury (Hoehn et al. 2001). In addition to neurons, endothelial cells also produce hsp72 (Gill et al. 1998, Wagner et al. 1999). Eberl and colleagues (1999) found that hsp72 is also increased in human umbilical vein endothelial cells (HUVEC) stressed with a tissue preservation solution commonly used in transplantation procedures. Consequently, detection of hsp72 expression allows us to compare isolated cultured endothelial cells with brain sections.

Unfortunately, many of the image analysis programs currently available are cumbersome, expensive, and their usefulness for evaluating cerebral injury at both the cellular and tissue levels is unproven. The purpose of the study reported here is to evaluate an image analysis software package for both immunocytochemical and enzyme histochemical methods. Our aim is to justify the use of a single computer program to evaluate samples more expeditiously and with less cost.

Material and methods

Rationale and determination of image analysis software

We chose the IPLab Spectrum 3.1f (Scanalytics, Vienna VA) because it is commercially available, user friendly, and inexpensive. Since it is one of the few Macintosh compatible systems originally available, we felt that many investigators would have ready access to this technology. The image analysis was performed using the standard software package and directions provided with the software. Threshold intensity values for enzyme histochemical evaluation of infarcts and immunocytochemistry were determined earlier using peak intensity values for the isolated individual color components.

MCAO stroke induction in rats

Sprague-Dawley rats weighing 300–400 g are subjected to a 2 h transient focal cerebral ischemia using the middle cerebral artery monofilament suture occlusion model in accordance with our institutional guidelines for the care and use of animals. At 24 h post-occlusion, the animals were sacrificed by anesthetic overdose and the brains removed for analysis.

Enzyme histochemistry: brain tissue sections

TTC staining

Brains were placed in cold physiological saline for 5 min and sliced into 5–3 mm thick sequential coronal sections using a rat brain matrix (ASI Instruments, Centerline MI). Sections were placed in tissue cassettes and stained by immersion in 0.2% TTC (Fisher Scientific, Pittsburgh, PA) at 37° C for 30 min in the dark. After staining, the brains were fixed in 4% paraformaldehyde for 2 days. Images of fixed stained sections were photographed, digitized, and processed for analysis using a CCD camera (Model DXC-151, Sony Corp. Park Ridge, NJ), with high resolution video (768 × 493 picture elements), frame grabber, and IPLab Spectrum 3.1f.

Image analysis of TTC staining

Hand-drawn calculations for infarct area, right and left hemisphere brain areas, and total brain area were generated by tracing around the unstained tissue on the digital image. In cases of noncontiguous infarcts, the separate areas were traced individually and added together. Rostral and caudal aspects of the brain slices were imaged and analyzed. Brain volumes were calculated from

averages of the surface area of the matching sides and the slice thickness. End sections were calculated using the approximate volume of a cone with the perimeters as the base and the thickness as the height.

IPLab Spectrum 3.1f determined the total and average areas of all segments that passed the preset color threshold criteria in the region of interest (ROI). The computer program discriminates the infarcted from non-infarcted tissue (threshold values were set at 63 and 116, respectively) and places an overlay on pixels corresponding to infarcted tissue. The program then was used to evaluate both total cross sectional area of each brain slice and infarcted area.

Infarct and brain volume data were determined by multiplying the cross-sectional area (computed using IPLab Spectrum 3.1f) by the thickness of the brain slice using Microsoft Excel (Microsoft Corp., Seattle WA). The infarct size was calculated as total infarct volume, percentage of total brain volume, and percentage of infarcted tissue relative to the corresponding contralateral cerebral hemisphere.

Immunocytochemistry: cells

Hsp72 generation in HUVEC

HUVEC (Clonetics, BioWhittaker) were seeded in 24-well plates and allowed to grow to confluence in endothelial growth medium (EGM-2; Clonetics, BioWhittaker). To stress the cells, culture wells were heat-shocked by substituting preheated 43° C medium followed by further incubation at 43° C under standard tissue culture conditions for 0, 1, 2, or 3 h. After incubation at 43° C, some cells were incubated further at 37° C for 1, 2, or 3 h. All cells then were fixed in 4% paraformaldehyde for 10 min at room temperature.

Immunocytochemical detection of hsp72 using fast red staining

Following fixation, HUVEC were rinsed in 1 × Tris-buffered saline (TBS) and incubated at 25° C for 30 min in 1X TBS with 1% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO) and 0.05% Tween 20 (Fisher Scientific). The cells were blocked sequentially with normal rabbit serum for 20 min and avidin-biotin blocking system (DAKPO, Carpinteria, CA) for 10 min at 25° C. After washing, the cells were incubated overnight at 4° C with the monoclonal hsp72 antibody (Stressgen Biotechnologies, BC, Canada) diluted 1:200, rinsed in TBS, and incubated with avidin-biotin-phosphatase complex (DAKO) for 45 min at room temperature. Hsp72

was visualized using 1 mg/ml of fast red (DAKO). Cells were counterstained with Harris' hematoxylin and enhanced using saturated lithium carbonate. Wells were sealed using Crystal Mount (Biomedica Corp, Foster City, CA) aqueous mounting medium.

Image analysis of fast red staining

Ten regions of interest from HUVEC monolayers were scored subjectively by the experimenter for percentage staining and staining intensity using values ranging from 0 to 3. Zero represented no staining, 1 represented less than 30% of the cells stained or faint staining intensity, 2 represented 30–60% of the cells stained or intermediate staining intensity, and 3 represented maximum staining in over 60% of the cells. The score was expressed as the mean of the 10 regions.

Stained cells were quantified with IPLab Spectrum 3.1f as the percentage of the area of the region of interest using the color segmentation command to analyze the red color component of the image. The minimum and maximum intensity threshold values are set at 164 and 218, respectively. The stained surface area was computed and normalized by dividing the computer-generated stained area by the number of cells in the region of interest. Staining determined by computer was compared to subjective assessment.

Immunocytochemistry: tissues

Stroke induction using MCAO in rats

Sprague-Dawley rats were subjected to a 2 h MCAO, described above. After 24 h, rat brains were fixed by transcardiac perfusion of 100 ml saline followed by 300 ml 4% paraformaldehyde at 25° C.

Hsp detection in rat brains

Rat brains were removed as described above, and placed in 4% paraformaldehyde for 1 h at 25° C. They were cryopreserved at 4° C for three days by placing brains sequentially in 10, 20, and 30% sucrose solutions, and frozen at –80° C. Frozen brains were cut into 30 µm sections using a cryostat (Leica Microsystems, Bannockburn, IL). Slices were incubated for 30 min in 1 × TBS, 1% BSA and 0.05% Tween 20 (TBST) at 4° C, and blocked sequentially with 0.6% peroxidase/methanol and an avidin-biotin blocking system (DAKO) for 10 min at 25° C. The sections were stained for hsp72 using monoclonal hsp72 antibody (Stressgen Biotechnologies) and a rat-absorbed biotinylated anti-mouse immunoglobulin (Animal Research Kit; DAKO). Sections

were incubated for 15 min in the primary/secondary antibody cocktail with blocking reagents. Antibody dilutions were 1:200 for hsp72 and 1:500 for the control β -tubulin III antibody (TuJ1, gift from Anthony Frankfurter, University of Virginia). After washing with buffer solution, streptavidin-peroxidase solution was applied for 15 min at 25°C, followed by 3,3-diaminobenzidine (DAB) staining. Brain slices were mounted on positively charged microscope slides using Crystal Mount (Biomed Corp).

Image analysis in rat brain

The stained areas of the rat brain were analyzed as described above. The minimum and maximum intensity threshold values were 129 and 255, respectively, to optimize the detection of the area outside the blue component of the image.

Statistical evaluations

Means were compared using a two-tailed Student's *t* test (Graphpad Instat V3.05, San Diego, CA). Graphpad Prism V3.0 was used for regression analyses. Pearson product moment correlation was also determined using Graphpad InStat V3.05.

Results

TTC enzyme histochemical staining

Figure 1 shows three photographs of the same coronal section of a rat brain subjected to middle cerebral artery occlusion (2 h ischemia and 22 h reperfusion) and stained using TTC. Frame A shows an infarct defined by the absence of the red color. In frame B, the same section can be seen with a hand-drawn outline of the infarcted area. Frame C shows the computerized color segmentation overlay (green) of the infarct area.

Table 1 compares the hand-calculated and computer-calculated values for infarct volume (mm^3), percentage of infarct, percentage infarct/contralateral hemisphere, and percentage of brain swelling for each rat. The mean values are 72.10 mm^3 for the hand-calculated measurements and 70.70 mm^3 for the computer-calculated measurements (depicted graphically in Fig. 2). The difference in infarct area means is less than 5% suggesting that there is no difference between computer- and hand-calculated values. The two values are highly correlated ($r = 0.9720$) and effectively paired ($\alpha = 0.05$, $P < 0.0001$).

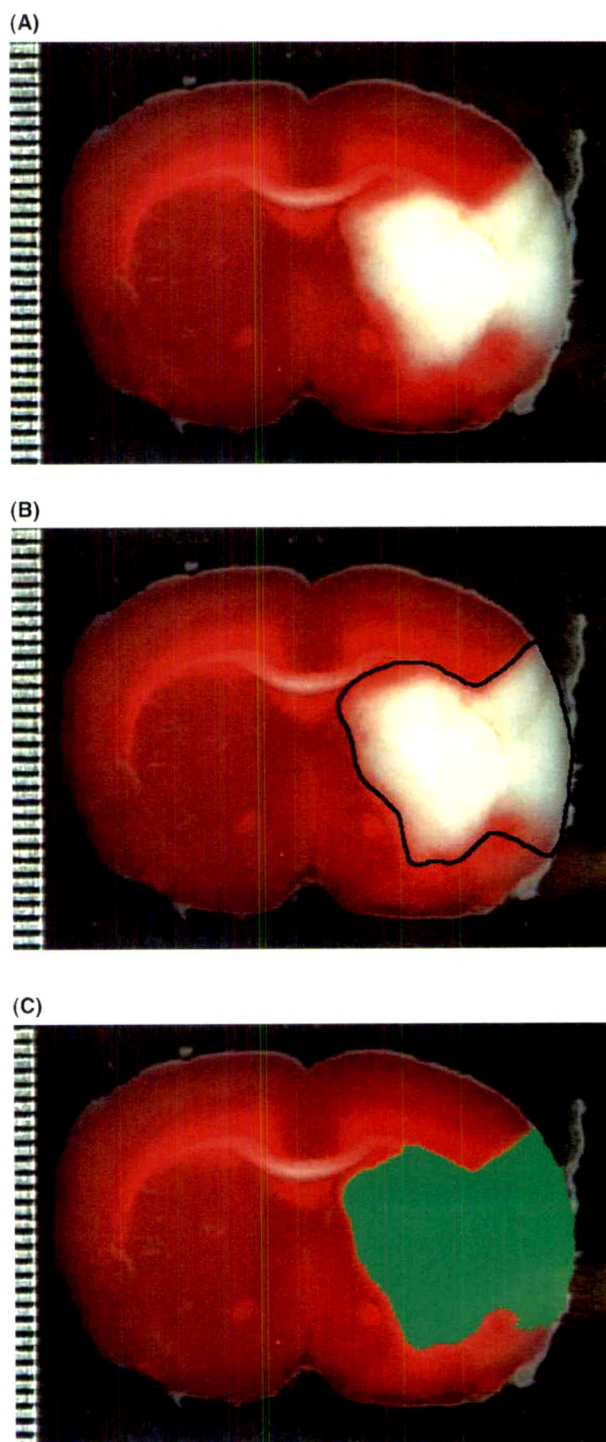


Fig. 1. Comparison of analytical methods for coronal sections of rat brain stained by TTC after MCAO. A) Cerebral infarct (white area) delineated by TTC following 2 h occlusion of the middle cerebral artery and 22 h reperfusion. B) Hand tracing of the infarct. C) Computer-generated assessment of the infarct (green). Interval between divisions shown on the left edge of each image is 0.0156 inch; 0.39 mm.

Table 1. Hand-calculated and computer-calculated values for infarct characteristics

Rat #	Hand-calculated value				Computer-calculated value			
	Infarct volume	% Infarct	% Infarct contrahemis	% Brain swelling	Infarct volume	% Infarct	% Infarct/ contrahemis	% Brain swelling
1	36.05	2.95	5.99	3.81	42.6	3.5	7.1	3.8
2	172.06	13.36	28.07	13.37	181.9	14.1	29.7	13.4
3	16.39	1.26	2.58	3.1	19.7	1.5	3.1	3.1
4	39.33	3.57	7.32	6.1	37.7	3.4	7	6.1
5	6.55	0.59	1.19	3.56	9.8	0.9	1.8	3.6
6	32.77	2.82	6.21	14.91	34.4	3	6.5	14.9
7	127.82	10.79	22.22	5.98	136	11.5	23.6	6
8	8.19	0.77	1.57	4.4	9.8	0.9	1.9	4.4
9	44.25	3.06	6.26	2.55	57.4	4	8.1	2.5
10	237.61	17.41	37.18	11.79	178.62	13.09	27.96	11.79
Mean	72.102	5.658	11.859	6.957	70.792	5.589	11.676	6.959
SD	79.35	5.96	12.63	4.62	68.04	5.19	10.96	4.62
SEM	26.45	1.99	4.21	1.54	22.68	1.73	3.65	1.54

Fast red Immunocytochemical detection of hsp72

Subjective scoring is compared in Table 2 to computerized image analysis data for a time course of hsp72 expression using endothelial cells stained with fast red. For both the subjective and computerized assessment of hsp72, the 0 h and 1 h time periods produced little or no detectable expression, whereas later time periods showed increased expression. Pearson correlation analysis between the subjective scores and computer-generated areas resulted in a correlation of $r = 0.7727$ ($\alpha = 0.05$, $P < 0.0001$) indicating a significant positive relationship between both analysis methods (Fig. 3). As expected, the subjective grade and the computer-generated normalized percentage stain-

ing of ROI increased as the duration of heat shock at 43° C increased. For both the 1 h and 2 h heat shock time periods, the extra incubation at 37° C also increased the amount of hsp72 expressed (Table 2).

Figure 4 shows an example of endothelial cells stained with anti-hsp72. The left side of Figure 4 shows immunocytochemical staining under light microscopy; the right side shows the computer-generated image evaluation of the same areas. The computer overlays in frames A through C indicate a progressively greater area of stained cells.

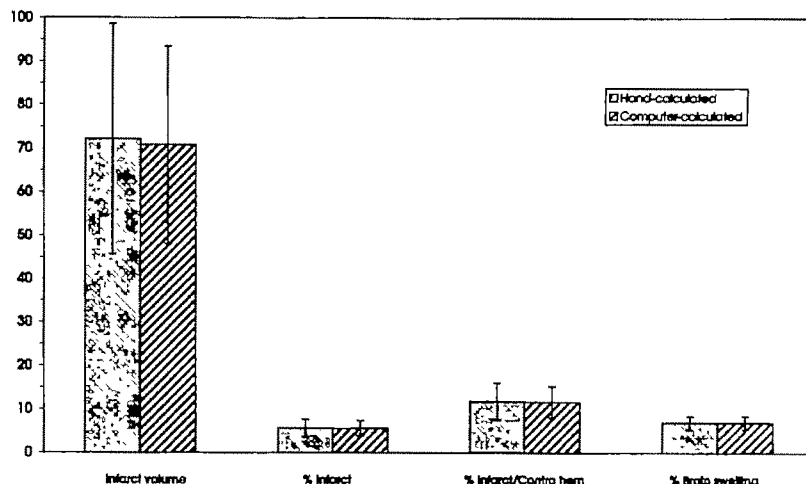
Hsp72 detection in rat brains following MCAO

Computerized image analysis was used to determine the area of hsp72 stained with DAB and

Table 2. Comparison of subjective grading and computerized percentage staining

Heat shock duration (h)	37° C Incubation after heat shock (h)	Subjective grade (0-3)	Staining in region of interest (%)	Cells in region of interest	Normalized staining (%)
0	0	0.5	16.6 +/- 5.9	90.8 +/- 17.1	0.18
1	0	1	10.4 +/- 8.5	109.7 +/- 23.4	0.09
1	1	1	39.8 +/- 18.4	103.6 +/- 23.3	0.38
1	2	2	66.9 +/- 17.5	102.8 +/- 19.5	0.65
1	3	2.7	69.6 +/- 13.8	107.8 +/- 22.7	0.65
2	0	0	6.2 +/- 1.1	135.8 +/- 24.3	0.05
2	1	1.5	30.2 +/- 16.0	139.9 +/- 13.4	0.22
2	2	2	46.5 +/- 13.9	129.7 +/- 12.7	0.36
2	3	2.1	61.3 +/- 6.8	113.1 +/- 21.1	0.54
3	0	3	86.2 +/- 3.4	119.3 +/- 14.1	0.72
3	1	2.5	80.7 +/- 5.7	150.5 +/- 21.9	0.54
3	2	3	86.0 +/- 10.2	101.2 +/- 12.3	0.85
3	3	3	88.3 +/- 2.6	103.1 +/- 10.7	0.86

Fig. 2. The means ($\pm$ SEM) for hand-calculated and computer-generated analysis for infarct area detected in rats subjected to MCAO compared graphically. There was less than 5% difference between two methods of analysis for infarct volume (mm^3), percentage infarct, percentage infarct relative to the contralateral hemisphere and the percentage brain swelling, suggesting that there is no difference between computer- and hand-calculated values. The two values are very highly correlated ($r = 0.9720$) and effectively paired ($\alpha = 0.05$, $P < 0.0001$).



counterstained with hematoxylin in rat brains. Hsp72 is localized in the cortical or penumbral region where neurons may survive the ischemic injury. Figure 5 shows coronal rat brain sections subjected to MCAO and stained for hsp72 as seen alone and with green overlaid, computer-assessed color segmentation. Hsp72 staining was defined by image analysis and determined to be 2.12% of the total brain area. The computer algorithm successfully captured the area stained by hsp72. Moreover, the computer can discriminate the stained and unstained background tissue.

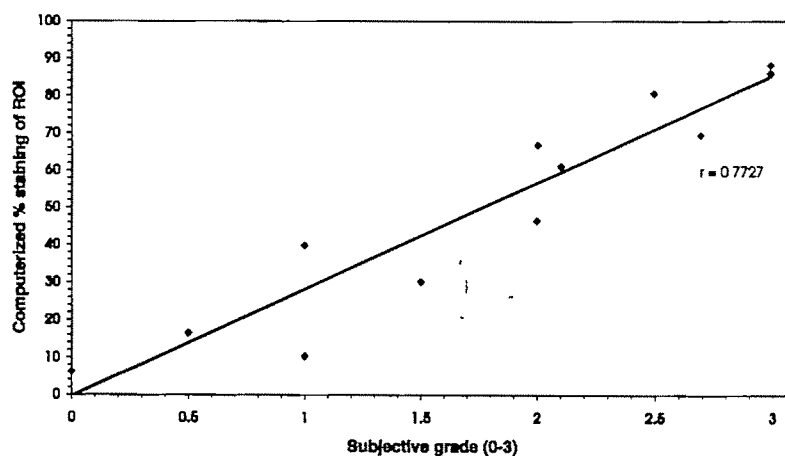
Discussion

Simple imaging systems often include a video camera, a frame grabber, a computer, and an image analysis program. They are useful tools for extracting specific information, such as light density, from an image. The video camera converts the light from the image into an electrical signal, which sends this signal to the frame grabber. A frame grabber is

essentially a board that digitizes the video signal. The software program is then able to process and manipulate the image so that the characteristics of the image can be analyzed. Current technology utilizing digital camera rather than frame grabbers is promising, but it is limited by the digital camera memory.

The primary aim of our study was to validate the use of a simple computer-assisted image analysis for both enzyme histochemical and immunocytochemical methods currently used to evaluate stroke. The data from our experiments indicate that image analysis provides an easy, efficient method by which to quantitate experimental differences assessed by various immunocytochemical and enzyme histochemical methods in both cells and tissues. In addition, the software's scripting features allow increased efficiency and ease of this computer-generated analysis. Computerized image analysis is also useful for detecting small differences in staining, especially where visual distinction may be difficult. This may occur when there are subtle

Fig. 3. Computerized image analysis of immunocytochemically stained HUVEC monolayers compared to a subjective grading system in the same experiments. HUVEC monolayers subjected to heat shock conditions were stained for hsp72 using immunocytochemistry and analyzed for percentage staining by computerized image analysis. Cells were also subjectively scored from 0 to 3 and compared using linear regression. Pearson correlation coefficient between the subjective scores and computer-generated areas resulted in high correlation ($r = 0.7727$, $\alpha = 0.05$, and $P < 0.0001$), indicating a positive relationship between the methods.



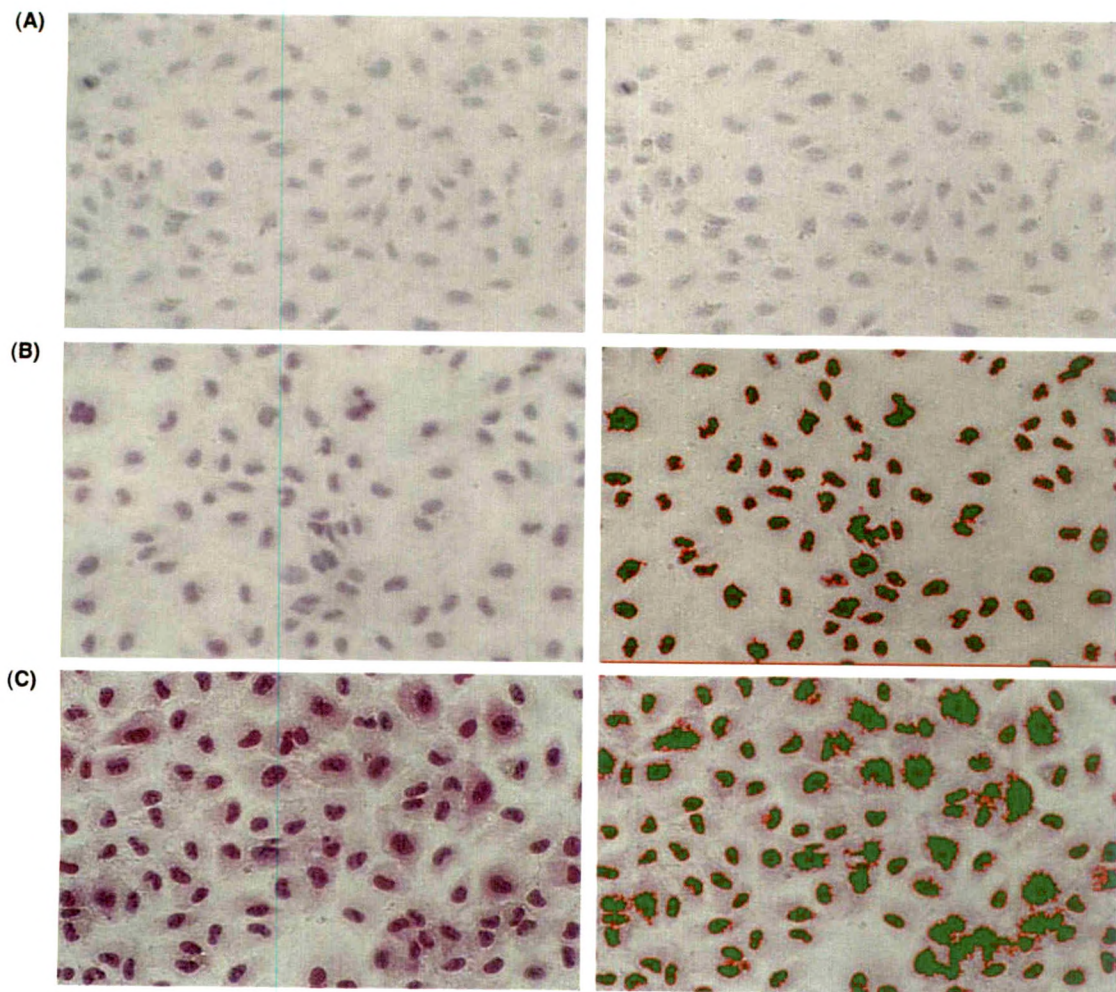


Fig. 4. Fast red staining for hsp72 of human umbilical vein endothelial cells following heat shock treatment. A) Control (0 h) on the left side with the associated computer-assisted image analysis overlay on the right side. There was no detectable staining for constitutively expressed hsp72. B) Immunocytochemical staining for the 2 h period and the corresponding image analyzed overlay. C) The 3 h period. Original photographs $\times 50$.

color intensity differences or when using dark stains such as DAB with hematoxylin counterstaining. Enhancing the edges of an image or eliminating background noise may allow the experimenter to discern subtle characteristics of an image. Perhaps the most important benefit of the image analysis system used here is that it can reduce investigator bias by predefining color threshold values and incorporating scripted routines.

Our results compare favorably with other studies of methods for quantifying stroke volume in experimental models. Three studies investigating quantitative analysis of stroke volume without computer assistance reported a significant problem with intermediate staining intensity at the border zone of the stroke (Liszczak et al. 1984, Hatfield et al. 1991, Dettmers et al. 1995). Our computer-assisted model uses predefined intensity thresholds that limit the effects of this intermediate staining

problem. A scanner-based image acquisition method showed similar results for TTC staining intact brain sections (Goldlust 1996). The image resolution of 300 pixels/inch is less than can be obtained by the CCD camera used in this experiment. Finally, Calloway et al. (2000) described a novel system for determining stroke volume based on light scatter dynamics. Although their method has advantages during the first 3 h of infarction, it requires construction of a special light-limiting box, and shows no advantage at the later time points evaluated in our study.

In our experiments, the IPLab Spectrum system was used to quantitate hsp72 in HUVEC and brain sections. In HUVEC, both subjective scoring and computerized analysis and the hand calculations were virtually identical in determining the percentage of staining and intensity; the use of time-saving scripted features makes the computer analysis a

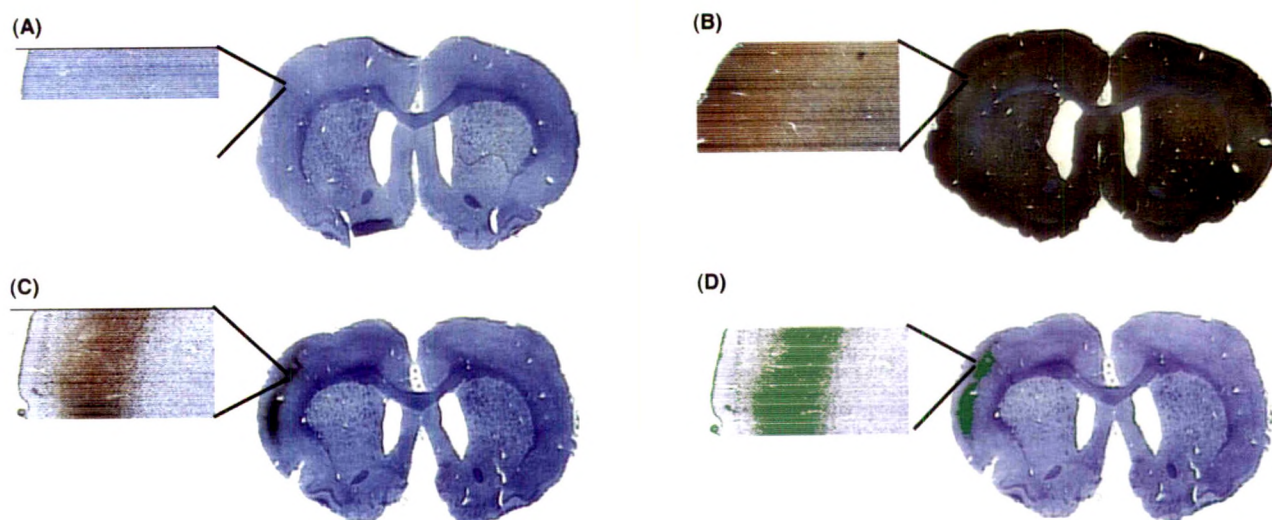


Fig. 5. Coronal sections of rat brain subjected to 2 h ischemia followed by 22 h re-perfusion, and stained using immunocytochemistry for hsp72 (DAB with hematoxylin counterstain). A) Negative control (no primary hsp72 antibody). B) Positive control (β -tubulin III Ab). C) Hsp72 staining in the ischemic penumbra. D) Green, color segmented overlay of hsp72 staining evaluated by image analysis. Whole brain coronal sections. Original magnification 10 $\times$; inserts; original magnification 100 $\times$.

more appealing option. This is supported by an earlier report using another cell culture system (Champelovier et al. 1991).

Limitations of image analysis software programs are of three general types: over-reporting data, under-reporting data and mis-reporting data. An advantage of the IPLab system is that by modulating the setting, over-reporting and under-reporting can be minimized. The correlation between the hand-drawn calculations and the IPLab system suggest that mis-reporting error is not a problem.

IPLab Spectrum image analysis software evaluation of TTC staining does not overcome the limitations of this staining method, as noted by Liszczak et al. (1984). Because TTC staining is based on intact mitochondria, areas of tissue with a low concentration of mitochondria may remain pale. In addition, the staining intensity of the TTC also decreases during 24 to 28 h fixation in paraformaldehyde, and may give erroneously low signal intensities. Improperly stained areas can cause an over- or underestimation of the infarct area by the computer depending on the pre-selected threshold values (Goldlust et al. 1996).

Another limitation in our experiment is the variability of staining of hsp72. Although variable staining has been reported in other studies, staining variability observed during physiological responses to cell stress limits the ability to compare different cell populations (Mailhos et al. 1994, Uney et al. 1993). By controlling the expression of hsp72 using transiently transfected HUVEC with a vector containing the hsp72 gene, whose expression can be

regulated in a dose dependent manner, we can compare more accurately computer-determined areas of staining with the actual amount of hsp72 expression induced by the drug.

Despite our validation of this simple, analogue based, computerized image analysis, new technology has overcome some of the limitations of the older systems. For example, the resolution of the digital image in the older system is limited by the resolution of the camera and the grabber technology. CCD cameras are configured to look at a small illuminated area, then to divide this area into pixels that can then be evaluated (Grant 2001). Alternatively, newer confocal laser scanners can provide a high pixel resolution by focusing light to a single point that approximates the size of a pixel (25–100 μ m). This method maximizes the number of significant pixels, thus improving picture quality and quantitative analysis. Other new image mapping strategies use more complex algorithms to visualize patterns in three dimensions. An advantage of these methods is that they allow direct calculation of brain volume. They also account for the contours of the image, and produce a more accurate calculation (Petroll et al. 1996). The main disadvantage of these new techniques is that it can still be very costly. In addition, the added sensitivity and discrimination may not be necessary for all applications. For a variety of applications, the simple computerized image analysis system is still valuable and cost effective.

The data reported here show that our image analysis system can be used to quantitate hsp72

staining in both rat brains and HUVEC monolayers. We also demonstrated that enzyme histochemical staining with TTC in rats following MCAO produced infarcted areas that can be defined and quantitated using an image analysis system.

Computerized image analysis offers better discrimination of cells and tissues stained using various enzyme histochemical and immunocytochemical staining techniques. It is more efficient than either hand calculations of stained areas or subjective grading of staining intensities. It reduces investigator bias, is easy to use, can be scripted to allow rapid analysis, and provides these capabilities at a relatively low cost.

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Human pineal luteinizing hormone receptors

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Abstract

The presence of luteinizing hormone receptors in human pineal glands from five females and three males, ranging in age from 61–89 yr, was examined by in situ hybridization and immunocytochemistry. The results demonstrated the presence of these receptors at the mRNA and protein levels in all the pineal glands examined. Pineal gland luteinizing hormone receptors could potentially be involved in the regulation of melatonin synthesis.

Key words: human pineal, immunocytochemistry, in situ hybridization, LH/hCG receptors

Luteinizing hormone (LH) and human chorionic gonadotropin (hCG), once thought to regulate only human gonadal functions, have now been found also to regulate the functions of many other organs in the body (Rao 2001) including brain (Lei et al. 1993). Previous studies have demonstrated that a common receptor, which mediates the actions of both LH and hCG, has been found in several regions in fetal (Al-Hader et al. 1997a,b), neonatal (Al-Hader et al. 1997c) and adult brain (Lei et al. 1993). The regions in adult brain include hippocampus, cerebellum, brain stem, choroid plexus, ependymal cells, hypothalamic gonadotropin releasing hormone (GnRH) neurons, and so on (Lei et al. 1993). LH and hCG have behavioral, neurotropic and neurotransmitter effects and regulate neuroendocrine activity (Al-Hader et al. 1997b,c; Toth et al. 1994; Lukacs et al. 1995; Lei and Rao 1994, 1995). Studies using LH receptor antisense oligodeoxynucleotides indicated that brain effects of LH require its receptors (Lei and Rao 1994, Zhang et al. 1999). Two previous immunocytochemical studies using antibody to LH claimed that human

pineal, a part of the brain, also contains LH receptors (Luboshitzky et al. 1997a,b). Because the results of these studies indicate the presence of LH immunoreactivity and not its receptors (Rao 1997), we investigated whether human pineals contain LH receptors by using in situ hybridization and immunocytochemistry using LH receptor antibody.

Materials and methods

Entire pineal organs were removed intact from human cadavers at autopsy (Table 1). All procedures were in accordance with the guidelines of the Human Studies Committee of University of Louisville. In several cases the cadaver was lightly embalmed and the pineal was removed after a day or two. Immediately after removal, pineals were drawn using camera lucida, divided into several parts, for fixation either in 3% glutaraldehyde or 10% neutral buffered formalin, dehydrated and embedded in paraffin. Five-micron thick sections were cut from each paraffin embedded tissue block.

A nonradioactive method of in situ hybridization was used for LH/hCG receptor mRNA detection in human pineals. Briefly, fluorescein-UTP (uridine triphosphate) labeled riboprobes were transcribed from human LH/hCG receptor cDNA obtained from Dr. Aaron J. W. Hsueh, Stanford University School of Medicine, Palo Alto, CA. Pineal sections were treated for 30 min at 37°C with 5 µg/ml proteinase K in 50 mM Tris-HCl, 5 mM EDTA, pH 7.5. For prehybridization, a mixture containing 50%

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Table 1. Human cadaver data relevant to pineal LH/hCG receptors

<i>Specimen no. Identification no.</i>	<i>Age, (yrs)</i>	<i>Sex</i>	<i>Immunostaining intensity</i>	<i>Date of death, postmortem/ embalming interval and cause of death</i>
1 (898)	68	F	++++	3/16/99, 3 days*, myocardial infarction, hypertension, diabetes mellitus
2 (900)	80	F	+/-	3/18/99, 5 days*, respiratory failure, chronic obstructive lung disease, emphysema
3 (833)	67	M	+++	5/2/89, 1 day, cancer
4 (896)	89	F	++	3/13/99, 12 days, cardiorespiratory arrest, senile dementia
5 (116)	76	M	++++	6/20/92, about 7 years, congestive heart failure, coronary pulmonary arrest
6 (1139)	61	F	++	12/9/00, 6 days*, cerebrovascular disease
7 (1130)	79	F	+++	11/25/00, 23 days*, coronary artery disease
8 (1167)	76	M	++	2/9/01, 8 days*, cardiopulmonary arrest

*The body was kept at 4° C until lightly embalmed within 48 h.

formamide, 5 × SSC, 1 × Denhardt's, 1 mg/ml yeast tRNA, 100 µg/ml heparin, 5 mM EDTA was applied to the sections and incubated for 3 h at 55° C in moist chambers. The hybridization was carried out overnight in the same mixture containing 200 ng/ml labeled receptor riboprobes.

Hybridization with sense probe served as a procedural control. After hybridization, the sections were washed at 55° C with 1 × SSC, and then with 2 × SSC for 15 min each time and finally twice with 0.2 × SSC for 30 min each time. Hybridization signals were detected 18 h after exposure to anti-fluorescein alkaline phosphatase conjugate and 4-nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate.

Immunocytochemistry was performed by an avidin immunoperoxidase method (Lei et al. 1993). Pineal sections were incubated overnight at 4° C with a 1:350 dilution of polyclonal antibody, raised against a synthetic N-terminus amino acid sequence of 15-38 of LH/hCG receptor, obtained from Dr. Patrick Roche, Mayo Clinic, Rochester, MN. For the procedural controls, either the receptor antibody was preabsorbed with excess receptor peptide or unabsorbed receptor antibody was replaced with nonspecific IgG. The immunostaining intensities were scored visually from one plus (light) to four plus (strong).

Results

Representative photographs of in situ hybridization and immunocytochemistry are presented here. The presence of LH receptors in human pineal glands was first investigated by in situ hybridization. The use of antisense riboprobe revealed dense hybridization signals in pinealocytes (Fig. 1A, B). The use of sense riboprobe for a procedural control produced no signals (Fig. 1C).

Immunocytochemistry using a polyclonal LH receptor antibody demonstrated receptor immunostaining in pinealocytes (Fig. 2A, B). Preabsorption of receptor antibody with receptor peptide produced complete absence of immunostaining (Fig. 2C). All pineal glands were immunopositive. Immunostaining intensity varied among specimens, however, and does not seem to depend on age, sex, time postmortem, cause of death, or method of tissue preservation and fixation (Table 1).

Discussion

LH/hCG receptors are single chain transmembrane glycoproteins that belong to the G-protein coupled receptor superfamily (Loosfelt et al. 1989, McFarland et al. 1989). They contain a large extracellular hormone binding domain and a seven

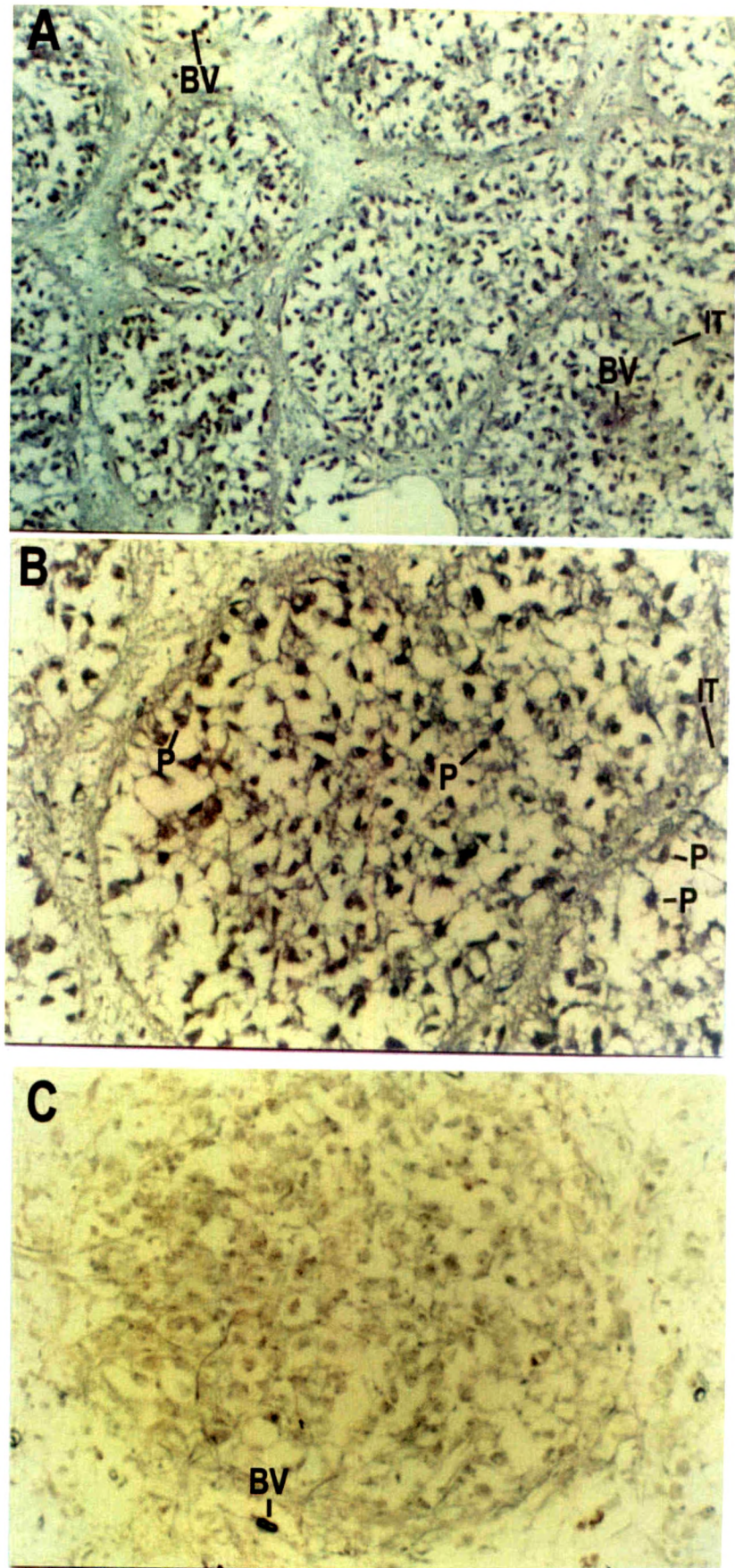


Fig. 1. In situ hybridization for LH/hCG receptor mRNA. Antisense riboprobe was used in panels (A) and (B) and sense riboprobe was used in panel (C). The hybridization signals with antisense probe, reflecting the presence of receptor mRNA, were detected in pinealocytes. BV, blood vessels; IT, interlobular trabeculae; P, pinealocytes. A $\times 150$; B and C $\times 300$.

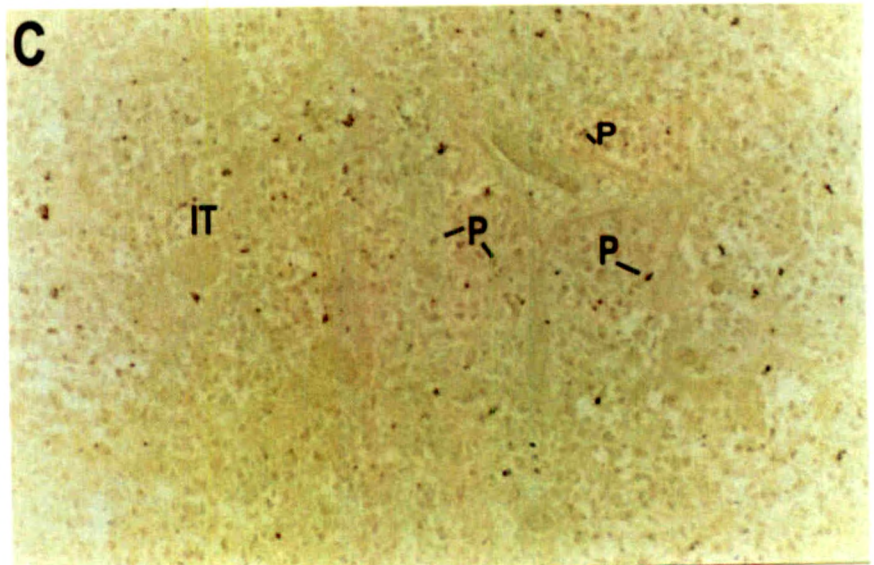
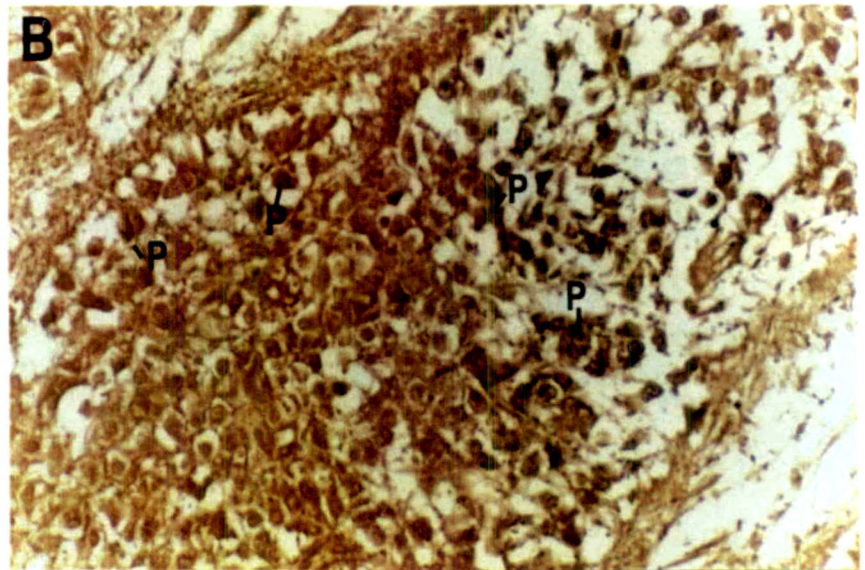
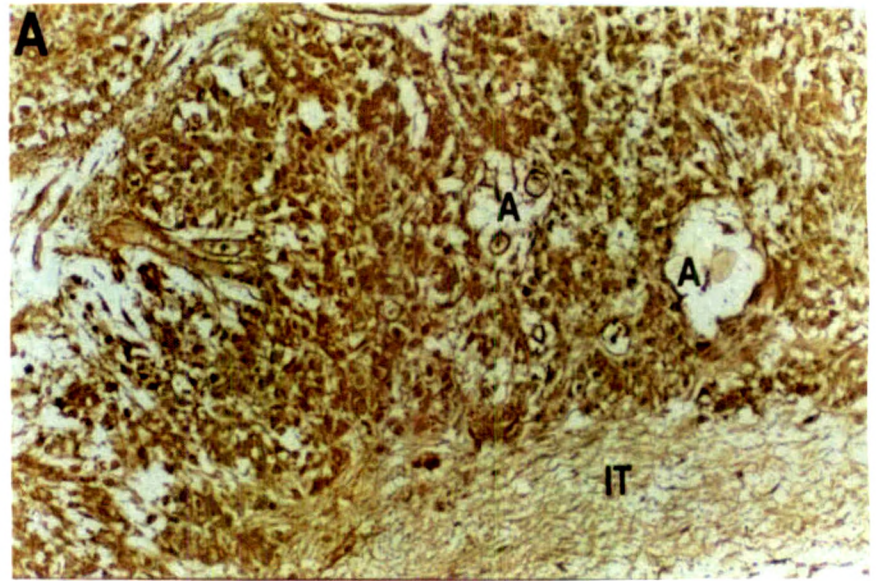


Fig. 2. Immunocytochemistry for LH/hCG receptor protein. Unabsoorbed receptor antibody was used in panels (A) and (B) and preabsorbed receptor antibody in panel (C). The receptor immunostaining was most abundant in pinealocytes. A, acervuli; IT, interlobular trabeculae; P, pinealocytes. A and C $\times 150$; B $\times 300$.



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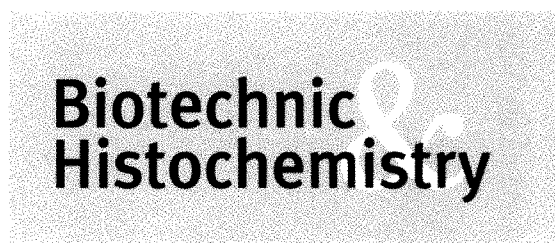
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transmembrane spanning region connected to a small intracellular domain, that is coupled to G-proteins (Loosfelt et al. 1989, McFarland et al. 1989). These receptors are encoded by a single gene containing 11 exons and 10 introns (Dufau, 1998). The ovary and testis are classical targets of LH and hCG actions where these hormones increase the production of steroid hormones and other regulatory molecules (Pierce and Parsons 1981). Studies published during the last 15 years have demonstrated that these hormones are also capable of regulating the functions of many nongonadal reproductive and nonreproductive organs (Rao 2001) including brain (Lei et al. 1993). The previous study on brain did not examine pineal glands for the presence of LH/hCG receptors. Meanwhile, Luboshitzky et al. (1997a,b) claimed that they identified LH receptors in human pineal glands using an antibody to LH. This antibody detects LH, but not its receptors. Therefore, our study demonstrating LH receptor transcripts and receptor protein in pinealocytes, is the first to document LH receptors in human pineal glands.

There is no apparent trend for receptor staining intensity related to gender, age, or specimen storage or processing. The number of samples analyzed was rather small, however, and further studies with a larger series are needed. Future studies will be needed to determine whether pineal gland LH receptors show seasonal variations, and diurnal differences.

The presence of LH receptors in human pineal glands does not necessarily mean that they are functional or that LH plays an important role in pineal gland functions. The previous studies give potential clues, however. For example, pineal glands play an important role in reproduction (Aleandri et al. 1966, Brzezinski and Wurtman 1988, Cardinali and Vacas 1978, Reiter 1998), GnRH is present and can regulate protein synthesis in pinealocytes (Halder-Misra and Pevet 1983), and gonatotropins along with gonadal steroids may regulate melatonin synthesis and other pineal functions (Cardinali and Vacas 1978, Cardinali et al. 1987, Luboshitzky et al. 1997a,b, Smith et al. 1975). Considering all these findings, it is not unreasonable to speculate that pineal gland LH receptors could be involved in the regulation of melatonin synthesis.

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Notes and queries

This section contains anonymous questions and signed, peer-reviewed answers to questions on topics within the scope of the journal. Send questions or answers by mail to: Dr JA Kiernan, Department of Anatomy and Cell Biology, The University of Western Ontario, London, Canada N6A 5C1, or by e-mail to kiernan@uwo.ca.

Safe diaminobenzidine (DAB) disposal

DAB (3,3'-diaminobenzidine hydrochloride), used to localize peroxidase-labeled antibodies and probes, is described as "possibly carcinogenic" in some books and articles. We must assume it is hazardous, so what is a safe way to dispose of used solutions?

This procedure, similar to that of Lunn and Sansone (1990), is the one approved by our state water department for disposal of used DAB waste from our Dako autostainer. You might need to check with your own water department before using it.

1. Prepare the following stock solutions:
0.2 M Potassium permanganate (31.6 g KMnO_4 /liter)
2.0 M Sulfuric acid (112 ml concentrated acid/liter)
2. For each 10 ml of working DAB solution, add:
5 ml 0.2 M Potassium permanganate
5 ml 2.0 M Sulfuric acid
3. Allow the mixture to stand for at least 10 h; this will allow the DAB to become noncarcinogenic.
4. To decolorize the solution, add powdered ascorbic acid until the color disappears.
5. Neutralize the decolorized solution with sodium bicarbonate. Test the neutrality (pH meter, pH paper or dipsticks).
6. Discard by pouring down the drain with copious amounts of running water.

If anyone has a simpler method we would love to have it. By the way, our water department will not allow us to use the procedure using sodium hypochlorite (bleach) to destroy DAB.

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Longitudinal sections of unfixed muscle

I have no trouble with transverse cryostat sections of unfixed skeletal muscle, but longitudinal sections frequently show severely damaged fibers. How can this be prevented? It does not happen with frozen sections of fixed muscle.

Frozen skeletal muscle retains the capacity to contract after it thaws. This can lead to problems when examining longitudinal cryostat sections. The tissue literally tears itself apart as portions of the muscle retract toward the areas of the section that first become attached to the microscope slide. No further contraction occurs with drying prior to histochemistry; the damage has already been done. Microscopically, this damage appears as multiple transverse tears in the myofibers, with the broken ends severely retracted. In areas where the damage is not so severe, partial tearing is apparent within the sarcolemmal sheath.

Muscle requires calcium ions (Ca^{2+}) to contract. A chelating agent such as ethylenediaminetetraacetate (EDTA) can combine with Ca^{2+} , incorporating the ions into a stable, biologically unreactive complex $[\text{Ca}(\text{EDTA})]^{2-}$ ion. EDTA can be used to prevent contraction in unfixed sections of skeletal muscle. The following pretreatment is applied to the glass slides before using them to collect sections from the cryostat:

1. Use only clean and grease-free slides.
2. Dip the slides in a 3% w/v solution of Na_2EDTA in distilled water for 5–10 sec. (Na_2EDTA is ethylenediaminetetraacetic acid disodium salt, dihydrate.)
3. Drain well and wipe excess Na_2EDTA solution from the backs and sides of the slides.
4. Allow the slides to air dry at room temperature in a dust-free environment.

Notes:

1. Use only slides with a uniform coating of EDTA.
2. It may be necessary to increase incubation times.
3. EDTA can be washed out of the sections by rinsing the slides in buffer for 15 min before proceeding with enzyme histochemistry.
4. If a calcium-activated ATPase technique is to be performed, it is necessary to rinse the slides in barbital buffer, and pre-incubate in 0.04 M calcium chloride in barbital buffer for 15 min. It is also necessary to increase the concentration of calcium ions in the ATPase substrate medium to 150% of the original concentration.

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Color preservation in pathology museum specimens

Is there a way to fix a large specimen (such as a heart or a slice of whole human kidney) so that the color will not be lost during long storage in a museum jar? The fixation must also be suitable for microscopic study of small samples taken from the same specimen.

Three steps are involved in preserving a pathological specimen for museum display: fixation, color preservation or restoration, and specimen preservation.

Fixation prevents autolysis and putrefaction and maintains the structural integrity of the specimen. After initial fixation in phosphate-buffered formaldehyde and collection of small pieces for histological study, the large specimen is placed for an extended period in a museum fixative formulation, either Wentworth's or Kaiserling's (Legault and Huang 1979, Daws 1996). Wentworth's fixative is the easier to prepare and use (Drury and Wallington 1967):

Formalin (37%): 100 ml

Sodium acetate: 40 g

Distilled water: 900 ml

Replace the fixative if it becomes discolored. It is essential that the tissues be fully fixed (usually 3–4 weeks) in this fluid.

The natural color of animal tissues is largely due to hemoglobin (red blood) or myoglobin (pink muscle). Fixation in formaldehyde immobilizes these

chromoproteins within cells, but with prolonged storage they degrade and become brown.

The simplest method for restoring color to specimens is that of Kaiserling (Drury and Wallington 1967), which uses controlled treatment with ethanol. Fixed specimens are placed in 80% ethanol for 30 min to a maximum of 4 h until the change in color from brown to red is at its maximum. Care is needed because prolonged treatment with ethanol can result in irreversible bleaching of the specimen.

The final step is preservation and mounting of the specimen. Most techniques use a 20–40% aqueous glycerine solution containing formalin (less than 1%) or thymol as an antimicrobial preservative (Edwards and Edwards 1959, Drury and Wallington 1967, Daws 1996). Sodium acetate (10%) and dibasic sodium phosphate (*ca.* 0.1%) are often included to prevent acidification; the pH should be 7.5–8.0. Sodium dithionite (3 g/kg of tissue) may be added to prevent oxidation and to maintain specimen color.

Problems associated with this method include lowering of the fluid level owing to water loss from leaky or porous plastic containers and leaching of tissue pigments (e.g., blood and bile) into the fluid. Israel and Young (1978) recommend using liquid paraffin (mineral oil) as a preservative solution. Tissues are removed from the 80% ethanol, rinsed quickly in absolute ethanol, blotted dry, and placed in the oil. The resulting specimens are brilliant, with no leaching of pigment from the tissues.

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Collagen type I staining

What is the most reliable antibody for detecting type I collagen in human and mouse tissue?

If you want to save time, effort and money, why not try staining with picro-sirius red? The method, introduced by Puchtler et al. (1973), is no more difficult to do than a van Gieson's stain. The reagents are inexpensive, and no harsh treatments such as antigen retrieval are needed.

Stain hydrated sections for 30 min in:

Sirius red F3B (CI 35780; Direct red 80): 0.1 g
Saturated aqueous picric acid solution: 100 ml
(This solution can be kept and used repeatedly for at least 4 yr (Kiernan 1999)).
Rinse in acidified water (0.1% acetic acid),
dehydrate in 3 changes of 100% alcohol, clear
and cover.

With this technique, all collagen is stained red, but only Type 1 is birefringent with crossed polars (Junqueira et al. 1979). The yellow and orange fibers stand out brightly on a dark background. The thinnest (reticular) fibers have green birefringence. It looks like fluorescence, but it never fades and the slides can be kept forever. Basement membranes, in which the predominant collagen is type IV, are stained red, but are not birefringent.

If picro-sirius red is preceded by nuclear staining with Weigert's iron-hematoxylin, this should be overdone, because much of the color is extracted from nuclei during the 30 min immersion in picric acid solution.

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Processing dried out specimens

I have been asked to examine sections of dried fish (salted and unsalted) by light microscopy. How should I set about processing to obtain paraffin sections?

Zimmermann (1976) showed that dried out fixed tissues could be rehydrated for histopathological examination by soaking in Ruffer's solution. This mixture was devised originally to enable the microscopic study of tissues from ancient Egyptian mummies (Ruffer 1921).

Ruffer's solution:

Water	50 ml
Ethanol (100%)	30 ml
5% Aqueous sodium carbonate	20 ml
(Alternatively, dissolve 0.6 g sodium carbonate in 42 ml water and add 18 ml alcohol, to obtain 60 ml of solution.)	

Rehydrate for 8 h or longer, depending on the size of the specimen, then proceed with dehydration, clearing and embedding.

At a National Society for Histotechnology Workshop, Zimmerman (1993) demonstrated rehydrated Egyptian mummy lung with flexible, normal looking tissue. The connective tissue framework, bacteria and parasites could be seen in appropriately stained sections, but nuclei and other intracellular structures were lost to autolysis. In contrast, normal and abnormal cells are quite well preserved in desiccated and rehydrated fixed specimens (Zimmermann 1976).

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Suppressing autofluorescence

What can I do to prevent or reduce troublesome autofluorescence in 40 µm frozen sections of formaldehyde-fixed brain that are immunostained with a fluorescein-labeled secondary antibody?

The traditional way to quench autofluorescence is to pretreat the sections with a solution containing heavy metal ions; osmium tetroxide is the most effective (Ornstein et al. 1957). Ornstein recommended 1% OsO₄ for 10 min, but a 0.2% solution for 5 min is also effective (Stoddart and Kiernan 1973). A long wash (at least 4 h in running tap water for slides bearing hydrated paraffin sections) is then needed to remove the OsO₄. Other techniques use certain dyes, including Sudan black B applied after immunofluorescence staining (Schnell et al. 1999, Baschong et al. 2001).

Recently, a method has been proposed that avoids the use of chemical quenching. The sections, mounted on slides, are irradiated for 12–48 h with visible and near-UV light from fluorescent tubes. The irradiation, which causes fading of the unwanted autofluorescence, is done before immunostaining. The original paper (Neumann and Gabel 2002) provides detailed instructions for this ingenious and simple procedure.

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Answers needed:

Is there a silver method for staining Alzheimer's neurofibrillary tangles that can be relied on to work every time?

Is it possible to obtain red staining of peroxidase-labeled secondary antibodies without using a proprietary product whose composition is a trade secret?

Book review

Principles and Techniques of Electron Microscopy, Biological Applications, fourth edition, MA Hayat. 543 pages. Cambridge University Press, Cambridge, UK, 2000

This beautifully printed new edition, to those experienced in the science and art of electron microscopy, will be like an old friend coming home, but even more prosperous than before and bringing specially selected gifts. Researchers, students, or technical staff who are skilled in the art, and beginners at least introduced to the techniques of electron microscopy and other forms of microscopy will find this book both pleasant and invaluable.

Fully revised and expanded, this fourth edition has new chapters that describe applications of microscopy using microwave technology, immunocytochemistry, antigen retrieval, and processing of plant tissues. A total of ten chapters thoroughly describe classic procedures in chapters entitled Chemical Fixation, Rinsing, Dehydration and Embedding, Sectioning, Support Films, Positive Staining, Negative Staining, and Low Temperature Methods. Each chapter is fully adequate and helpful. The chapter entitled Hazards, Precautions and Safe Handling of Reagents is up front as Chapter 1, and rightly so. The index is accurate and the text is well referenced.

Fortunately, in this careful compilation of electron microscopy methodologies, there are many

tried and true procedures to undergird voluminous research investigations. Some obsolete procedures appropriately have been dropped. Providing reliable and simplified recipes is a service additive to the biochemical concepts provided as a solid and necessary foundation for successful transmission electron microscopy. Procedures dedicated to scanning electron microscopy are only touched on and must be found elsewhere.

In the preface, which we often skip over, it is noteworthy that the author comments that, "During the last decade, although the basic principles have remained unchanged, significant advances have taken place in methodology for light, scanning and transmission electron microscopy", but also astutely notes that "progress in preparatory procedures to obtain optimal preservations of biological specimens has fallen far behind improvement in instrumentation." Perhaps this will inspire some creativity in that vein.

The author generously acknowledges our eminent colleagues who have contributed to the formation of this book and expresses his appreciation to them, as do I.

Beverly Giammara

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Stains recently certified

The batches of dyes approved since those listed in the last issue of this journal are listed in the table below. Certification does not imply approval for medicinal use.

Stains certified from April 1, 2002 through May 31, 2002*

<i>Name of dye</i>	<i>Certification number</i>	<i>Manufacturer and/or vendor</i>	<i>Date approved</i>
Aniline blue	DcK-8	Dudley Chemical Co.	May 23, 2002
Crystal violet	AbC-2	Abbey Color Inc.	Apr 18, 2002
Eosin Y	CcE-4	Carolina Color & Chem.	May 29, 2002
Eosin Y	DcE-24	Dudley Chemical Co.	Apr 22, 2002
Eosin Y	AcE-52	Aldrich Chemical Co.	May 29, 2002
Erythrosin B	DcEr-2	Dudley Chemical Co.	May 15, 2002
Hematoxylin	CcH-31	Carolina Color & Chem.	May 29, 2002
Hematoxylin	AbH-61	Abbey Color Inc.	May 29, 2002
Light green SF yellowish	AcL-22	Aldrich Chemical Co.	Apr 22, 2002
Methyl green	AcG-25	Aldrich Chemical Co.	Apr 22, 2002
Methylene blue	AcA-21	Aldrich Chemical Co.	Apr 22, 2002
Oil red O	AcRo-16	Aldrich Chemical Co.	Apr 24, 2002
Pararosanillin	DcFp-19	Dudley Chemical Co.	Apr 17, 2002
Phloxine B	Eph-39	Fisher Scientific Co.	Apr 19, 2002
Sudan IV	DcZ-5	Dudley Chemical Co.	May 29, 2002

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Oren N, Letenski MS (1991) Motor nerve terminal staining combined with catecholamine histochemistry or immunocytochemistry. *Biotechnic. & Histochem.* 66: 1-25.

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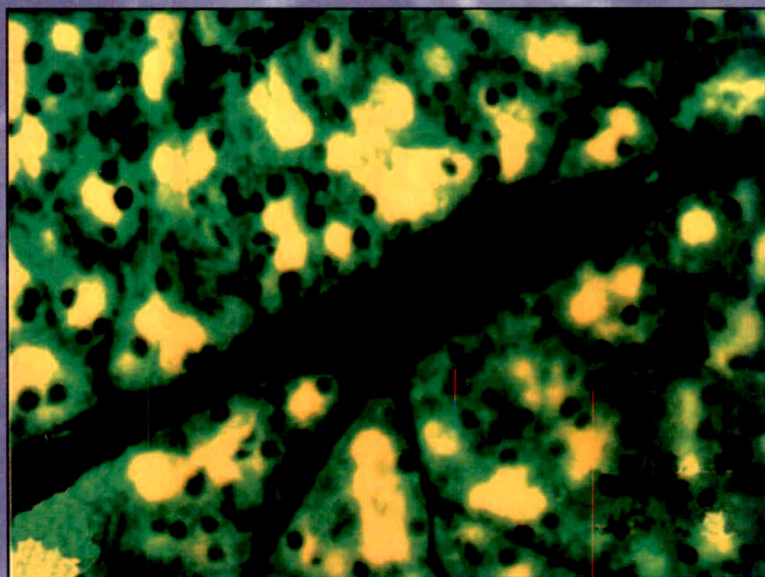
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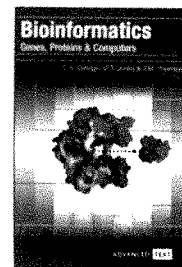
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ISBN: 1859960545 **Published:** Dec 02 **Price:** £29.99 **Pages:** 320

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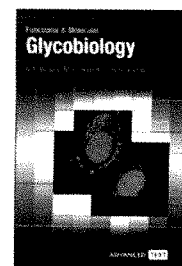
Functional & Molecular Glycobiology

S.A. Brooks, M.V. Dwek and U. Schumacher

Respectively School of Biological and Molecular Sciences, Oxford Brookes University, Oxford, UK; Department of Surgery, Royal Free and University College Medical School, London, UK and Institute for Anatomy, University Hospital of Hamburg-Eppendorf, Hamburg, Germany

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Functional and Molecular Glycobiology explains the chemistry of carbohydrates, and the biosynthesis and diversity of different types of glycans. It looks at the functional role of glycoconjugate compounds in normal cellular processes, in both disease and development. The book also describes how these compounds can be studied, analysed and manipulated for use in biotechnology. *Functional and Molecular Glycobiology* is a unique introduction to the field bridging the gap between introductory books and current research.

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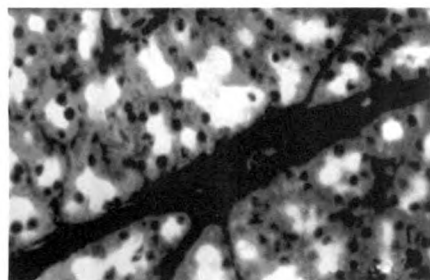
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Front cover: Epifluorescence microscopy showing H & E stained mouse pancreatic acinar cells.

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Analysis and testing of biological stains— The Biological Stain Commission Procedures

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Procedures for evaluating dyes as biological stains were developed for the most common stains and published by Peterson et al. (1933a,b,c, 1934a,b). These methods, which have been improved and extended over the years, are used for the examination of dyes and stains submitted to the BSC for certification.

The original methods included determinations of light absorption using a visible wavelength spectrophotometer. The most practical use of this instrument was to express the color characteristics of a sample by a simple ratio of color densities at two selected wavelengths. Later, the availability of a photoelectric spectrophotometer made it possible to characterize stains by their complete absorption spectra and by exact location of their absorption maxima. The same type of ratio has been retained, however, as additional characterization.

It has proved possible to assay by spectrophotometry the dye content of stains whose manufacturing procedures are apparently sufficiently standardized to result in closely similar spectral characteristics among different batches of the same dye. The color density at the maximum absorption of such stains is easily related to the dye content as determined by other methods. For many stains, however, samples show considerable variation in the position of the absorption peak, and it is not possible to perform an accurate spectrophotometric assay. Such dyes are assayed by volumetric titration with titanous chloride, or, in the cases of eosin and related xanthenes, gravimetrically after precipitation of the color acid.

The following is an account of the methods currently used in the Biological Stain Commission

(BSC) Laboratory in Rochester, NY. General remarks concerning methodology are followed by accounts of the assays and other tests for all stains certified by the BSC.

If the term alcohol is used, one may assume it refers to ethyl alcohol (ethanol), unless methyl alcohol (methanol) or isopropyl alcohol (isopropanol) is designated. Absolute alcohol is 100% or anhydrous ethyl alcohol; 95% alcohol (rectified spirit) has the highest concentration that can be manufactured by distillation of ethanol-water mixtures. Denatured alcohol (industrial methylated spirit) can be substituted for 95% alcohol in many applications, but is not recommended because of the variety of substances added to make it undrinkable (Budavari 1996). Unless otherwise described, the term water refers to distilled or deionized water. Where reference is made to formaldehyde fixation, it is assumed that the fixative is a "neutral" (pH 7.2–7.6) buffered solution containing approximately 4% formaldehyde derived from either dilution of formalin or depolymerization of paraformaldehyde.

General methodology

Spectral characteristics

A 50 mg sample of the homogeneous powdered stain is weighed using a Mettler Toledo balance. The entire sample is transferred to a 250 ml volumetric flask and approximately 225 ml of the appropriate solvent added. The flask then is shaken on an orbital shaker (VWR Scientific) for 20 min at 295 rpm to ensure complete solution of the dye. The solution is made up to the mark with solvent, thoroughly mixed, and an appropriate aliquot is taken for further dilution to provide a satisfactory dye concentration for spectrophotometric work.

Matched Pyrex cells of 1 cm light path are employed in any suitable manual or recording spectrophotometer. The reference cell contains the same solvent as the dye solution. At present, the

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BSC uses a Hewlett-Packard 8453 UV-VIS spectrophotometer. With manual spectrophotometers, readings are taken at least every 5 nm and usually at smaller intervals in the area of the absorption maximum. A reading at the exact peak is, of course, desirable for spectrophotometric assay.

The older method of expressing the shape of the curve by the ratio of color densities at two predetermined wavelengths loses its significance if the exact peak is known, because any difference in the position of the peak necessarily causes a change in the ratio. More elaborate methods have been used to express the shape of a curve independent of the peak position (Lillie and Roe 1942), but we have chosen the simple ratio of the color densities at minus and plus 15 nm of the peak wavelength ($P-15/P+15$) measured for a given sample. These densities can be estimated from the plotted spectrum or an absolute figure can be measured after the peak is located.

Titanous chloride assay

Many dyes are reduced by Ti^{3+} ions to colorless or differently colored substances. Ti^{3+} salts are readily oxidized to Ti^{4+} by atmospheric oxygen. Consequently, titanous chloride solutions must be standardized before use. This is accomplished by titration against a standard ceric sulfate solution. The BSC laboratory purchases a ready-made certified, standardized 0.1 N cerium(IV) sulfate solution, from Fisher Scientific Co. (Catalog No. SC66-1). This obviates the need in earlier times to make and standardize solutions of ceric sulfate. The $Ce(SO_4)_2$ solution is completely stable.

The indicator used for titration of $TiCl_3$ against $CeCl_4$ is ferrous-*o*-phenanthroline, which gives a sharp end point.

Standardization of titanous chloride solution

The stock solution is 20% titanous chloride stabilized with hydrochloric acid. An approximately 0.05 N solution is prepared by diluting 8.0–8.5 ml of the stock solution to 200 ml with water. Storage experiments with this diluted titanium solution showed that it can be stored under mineral oil for a normal working day without change, and for 20 h with a 1–2% decrease in strength. In practice the solution is placed in a burette equipped with a side reservoir, and both surfaces of the solution are covered with 13 mm of mineral oil. A fresh solution is made daily and the solution standardized against ceric sulfate solution (N/10) as follows. Exactly 5 ml of standard ceric sulfate are placed in a 50 ml Erlenmeyer flask, and 5 ml of concentrated sulfuric

acid added. After adding a drop of ferrous-*o*-phenanthroline indicator, the solution is titrated with the stored titanium solution. The initial yellow color of the solution passes through a green stage, then nearly colorless, and finally a single drop of Ti^{3+} produces the orange color of the reduced indicator. The titration is rapid and the end point exceedingly sharp.

Dye titration with titanous chloride

The dye titration procedures are based on those recommended in the 5th edition of *Biological Stains* (Conn 1946) with some minor modifications and improvements. Smaller amounts of dye and a more dilute titanous chloride solution are employed than in the original method. In most cases a bitartrate or tartrate buffer is included to stabilize pH during the titration. Sodium bitartrate, which is usually sold as sodium hydrogen tartrate (monohydrate), $NaHC_4H_4O_6 \cdot H_2O$ (FW = 190.08), provides an acidic medium (pH ~ 3.5). Sodium tartrate, more completely named sodium tartrate (dihydrate), $Na_2C_4H_4O_6 \cdot 2H_2O$ (FW = 230.08), provides mildly basic conditions (pH ~ 8.7). For some dyes, a concentrated acetate buffer is used instead. A 3.67 M stock solution of sodium acetate (111.6% w/w $CH_3COONa \cdot 3H_2O$) is mixed with glacial acetic acid: either 6 ml sodium acetate and 4 ml acetic acid for pH 4.0 or 8 ml sodium acetate and 2 ml acetic acid for pH 4.5. The 10 ml of concentrated buffer is added to the titration vessel.

To maintain anaerobic conditions and to minimize atmospheric oxidation of reduced dyes, the titration is conducted in an atmosphere of nitrogen. This gas is first washed by bubbling it through two bottles, each containing a solution of titanium chloride and safranin (Fig. 1). The following is a general description of the titanous chloride assay as carried out in the BSC laboratory. Specific variations will be noted under the individual dyes.

The titration is carried out in a Pyrex glass vessel with approximately 500 ml capacity with four ground glass openings for gas inlet, gas outlet, buret and thermometer (Fig. 1). The titration vessel is mounted on a magnetic stirrer hot plate with a Teflon coated magnetic stirring bar. Nitrogen is passed successively through two gas-washing bottles each containing titanous chloride and safranin. This washing solution is made by diluting 40 ml of 20% titanous chloride and 40 ml of concentrated hydrochloric acid to 500 ml with water, and after filling the gas-washing bottles, adding approximately 10 mg of safranin to each bottle. This mixture deteriorates in about 3 days without a change in color. It is therefore used only on the day

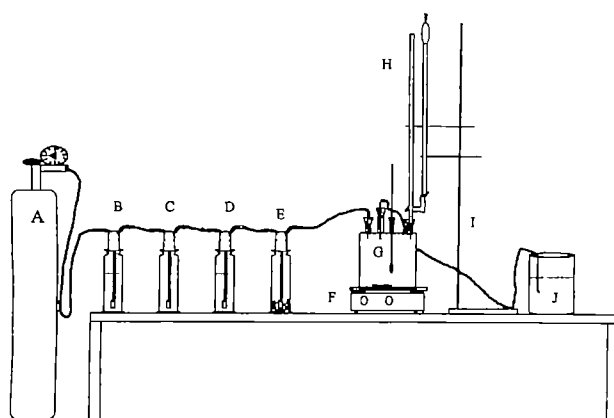


Fig. 1. Apparatus for titanous chloride titration. A) Nitrogen source (open valve to allow a steady flow of bubbles in the gas washing bottles B, C and D). A steady flow of bubbles in the collection beaker, J, verifies that the system is not leaking. B–D) Gas washing bottles, each two-thirds filled with TiCl_3 -HCl-safranin solution. E) Dry bottle with cotton in bottom to capture any fluids caught in tubing. F) Hot plate-magnetic stirrer. G) Custom made 500 ml titration chamber with 4 ports for buret, thermometer, gas inlet and gas outlet. H) Buret, filled with TiCl_3 solution. I) Ring stand. J) Washing beaker, 500 ml, filled with water to rinse exhaust gas from titration chamber.

it is prepared. The washed nitrogen then flows through the titration vessel.

The weighed dye sample is dissolved in the appropriate solvent in the titration jar, and buffer added. The total volume of dye mixture is usually 200 ml. The solution is stirred, heated if necessary, and the nitrogen passed through for at least 15 min before the titration is started. Like most titrations, the first run locates the approximate end point so that a second run can be rapid and the type of end point color change will be familiar.

The percentage of dye is calculated by the general formula:

$$\text{Percentage of dye} = \frac{\text{ml TiCl}_3 \text{ used} \times \text{normality of TiCl}_3 \times \text{MW of dye} \times 100}{\text{weight of sample} \times \text{no. of hydrogen equivalents} \times 1000}$$

The molecular weight of the dye is that of its organic ion with the usual counter-ion (usually Cl^- or Na^+) and does not include water of crystallization or other inorganic components such as half- ZnCl_2 .

Chromatography

The BSC employs chromatography to obtain both qualitative and quantitative analyses of biological stains known or expected to contain two or more

colored components. The dyes routinely examined by chromatography include azure A, azure B, azure C, Giemsa stain, Jenner's stain, MacNeil's tetrachrome stain, methylene blue, methylene violet Bernthsen, oil red O, thionine, toluidine blue O and Wright's stain. Other dye samples are subjected to chromatography periodically as a method of quality control.

Two types of chromatographic analysis are used in the Assay Laboratory of the BSC: thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). For each of these procedures the dye sample is completely dissolved in an appropriate solvent and placed onto stationary phase material. The dye molecules are moved through the stationary phase material by a mobile phase solution or running solvent. Different molecules move at different speeds owing to variations in their polarities. This allows a chromatogram to be generated from a dye mixture. Owing to the complexity of chromatography and the variety of methods used to separate different dyes, only an overview of the procedures is provided here.

TLC

TLC is a method for examining the composition of a sample by separating its components on a thin layer of stationary phase material, which is usually spread over a glass plate. Currently, we use reverse phase and Unisil silica gel plates purchased from Analtech (Newark, DE). The prepared dye solution is spotted on the plate in both 5.0 μl and 10.0 μl volumes. A control sample, whose composition is known, is also spotted on the same plate. The plate is then placed in a developing chamber in which the atmosphere is saturated with the vapor of the running solvent. The running solvent varies with the dye and the type of plate used. Currently, either toluene or an 8:1:1 water:methanol:1.0 N sodium hydroxide solution is used as the running solvent for reverse phase plates. A 10:2:1 *n*-butanol:water:glacial acetic acid mixture is used for the Unisil silica gel plates. The TLC plate remains in the chamber until the running solvent approaches the top of the plate and a clear distinction exists between spots on the plate. Typically this takes 40–90 min.

Once a plate has dried, the positions of the spots, or R_f values (R_f = distance migrated by spot/ distance moved by solvent front), are compared for qualitative analysis. Quantitative measurements are obtained with an LKB Bromma Ultrascan XL Laser Densitometer. This instrument measures the density of each spot, plots the position vs. density, then

calculates the proportion of the colored material at each spot.

HPLC

HPLC is replacing TLC for most of the dye samples analyzed by the BSC. Currently, the Assay Laboratory uses a Hewlett Packard Series 1050 HPLC system with HP Chemstation software. For HPLC analysis, 20 μ l of the prepared dye solution is introduced into an injector that leads to a column containing the stationary phase material. Currently, we use a reverse phase C-18 column (Vydac Hesperia, CA) that allows the dye components to separate based on the relative polarities of their molecules. The mobile phase of the HPLC system is pumped through the column under pressure, allowing rapid separation (< 12 min). HPLC grade acetonitrile and 0.062 M perchloric acid are used as mobile phase solvents for the reverse phase column. The mobile phase solvents are degassed by water-pumped helium at 30 psi. The composition of the mobile phase may be adjusted during separation to produce a gradient elution that further increases the separation of the dye components. As these components elute from the column, their concentrations are measured by a variable UV-visible detector set at an appropriate wavelength between 190 and 600 nm.

The HPLC system measures the time that the eluents are detected and the absorption of the eluted material. These data are then plotted as a series of peaks that show the absorption of each eluent and the time at which it was detected. This chromatogram qualitatively displays the composition of a mixture. The peaks are identified by comparison with chromatograms made from samples of known composition. Quantitative analysis is performed by calculating the percentage of each eluent based on individual peak areas in relation to the total area of all the peaks of a given sample. It should be noted that a percentage value obtained by chromatography is relative to the values of detectable components in the sample and is not the actual dye content.

General approach to examination of individual stains

Study of an individual stain by the general methods described above consists of measuring the spectra of a series of samples that have satisfactory staining quality. Ratios of the color density at the peak (D-peak) to previously recorded assay values are then calculated and compared. If these ratios (Assay/D-peak) are reasonably constant, the chemical assays

are carefully repeated, the ratios recalculated, and the average value used as the requirement for spectrophotometric assay of the stain. If the ratios are not reasonably constant, either the spectrophotometric data or the chemical assay must be considered unreliable. Spectrophotometric assay is, obviously, unsuitable when representative samples of a stain show great variation in the position of the peak or other spectral characteristics. In such cases the chemical assay by titration with titanous chloride is the method of choice. For a few dyes, the titanous chloride assay is not entirely satisfactory, and it has been difficult to establish percentage of purity. In some cases nitrogen analyses have been carried out and percentage of dye calculated on this basis. This figure in turn serves to standardize the spectrophotometric assay of the dye.

It should be noted that the methods reported here are designed to provide a quick chemical and spectrophotometric evaluation of commercial stains as they are received for certification. Therefore, the standards are based primarily on past commercial samples whose use in staining procedures is satisfactory. From a strictly chemical standpoint it is recognized that "percentage of dye" as determined by titanous chloride titration, spectrophotometry or color acid precipitation cannot adequately describe the true content of the dye under study, but in most cases it is very close.

Testing of individual dyes for certification

Qualitative and quantitative procedures for certification of dyes are, for the most part, the same as the general methods outlined above. Where special testing is required its description will be found under the heading for the individual dye.

Acid fuchsine (C.I. 42685)

Acid fuchsine should be the disodium salt of the trisulfonic acid of rosaniline or pararosaniline, or mixtures of these, and related dyes (Horobin and Kiernan 2002). Some samples may contain the mono- or disodium salts of disulfonic acids or the trisodium salt of the trisulfonic acid; when this is the case they are not nearly as satisfactory as those containing only the disodium trisulfonates. Unless the manufacturer has provided specific information about the character of the fuchsine, a sample of acid fuchsine is considered for analytical purposes to be a mixture of equal parts of trisulfonated rosaniline and pararosaniline.

Absorption characteristics

Dissolve 50 mg of acid fuchsin in 250 ml water. Dilute 5 ml of this solution with 5 ml 0.1 N HCl added to 200 ml with water. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 542–546 nm; P–15/P+15 ratio, between 1.10 and 1.26.

Assay

Dissolve 200 mg of dye in 50 ml 95% alcohol, add 150 ml water and 10 g sodium bitartrate ($\text{NaHC}_4\text{H}_4\text{O}_6 \cdot \text{H}_2\text{O}$). Heat to boiling and titrate with 0.05 N TiCl_3 to a yellow end point. The following data are used to calculate the percentage of dye in the original sample when calculated as:

1. The disodium salt of rosaniline trisulfonic acid:
Molecular weight 585.560
Hydrogen equivalents per mole of dye 2
Milliliters of N/10 TiCl_3 per gram of dye 34.155
2. The disodium salt of pararosaniline trisulfonic acid:
Molecular weight 571.533
Hydrogen equivalents per mole of dye 2
Milliliters of N/10 TiCl_3 per gram of dye 34.994
3. A mixture of equal parts of (1) and (2):
Mean molecular weight 578.546
Hydrogen equivalents per mole of dye 2
Milliliters of N/10 TiCl_3 per gram of dye 34.569

For certification, samples of acid fuchsin must contain $\geq 60\%$ dye.

Andrade indicator

Acid fuchsin is tested for its behavior in the Andrade indicator. A 0.2% solution of the dye in 100 ml water is decolorized by adding 1.0 N NaOH a little at a time. Complete decolorization should not require more than 5 ml of sodium hydroxide.

Biological tests

Van Gieson connective tissue stain. Acid fuchsin is tested in the Van Gieson stain on animal tissue fixed in formaldehyde or other suitable fixative and embedded in paraffin. The paraffin is removed and the sections are hydrated in the usual manner. The sections are stained for 2 min in Weigert's iron hematoxylin (see under hematoxylin below), washed in tap water, rinsed in two changes of distilled water, and stained for 3 min in Van Gieson's solution (made by adding 10 ml 1% aqueous acid fuchsin to 100 ml saturated aqueous

picric acid). The sections then are passed through three changes of 95% and three changes of 100% alcohol, cleared in three changes of xylene, and coverslipped using a resinous mountant.

The tissue elements stained by acid fuchsin (collagen fibers) should appear clear red, contrasting well with blue-black nuclei and yellow (picric acid) cytoplasm and erythrocytes. With a good sample of acid fuchsin, collagen stained by Van Gieson's method does not fade, even in slides stored for many years.

Mallory's connective tissue stain. Acid fuchsin is also tested in Mallory's connective tissue stain on animal tissue. Mallory's method involves the use of three dyes. The dye being tested is used in conjunction with already certified samples of the other two dyes. Suitable test objects are organs that contain basement membranes or thin collagenous fibers, such as kidney or intestine.

Specimens are fixed in Bouin's fluid (Zenker or Carnoy may be used instead) and embedded in paraffin. The paraffin is removed in the usual manner and the hydrated sections are stained in 0.5% aqueous acid fuchsin for 10 min or longer. The sections then are transferred directly (without washing) to a solution containing 0.5 g aniline blue and 2.0 g orange G in 100 ml 1% aqueous phosphotungstic acid. They remain in this solution for 20 min or longer; a wet slide is checked from time to time. When collagen fibers are blue and the cytoplasm of most cells, including muscle, is pink, the slides are rinsed briefly in water, dehydrated in three changes of 95% and three changes of 100% alcohol, cleared in xylene, and mounted in a synthetic resin.

A satisfactory sample shows red nuclei, pink cytoplasm, and blue collagen. Mucus and cartilage matrix are also blue. Erythrocytes and the cytoplasm of certain secretory cell types are orange or yellow.

Altmann's mitochondrial stain. Acid fuchsin is also tested in Altmann's mitochondrial stain (see under ethyl green below).

Alcian blue 8GX (C.I. 74240)

Alcian blue is a water soluble copper-phthalocyanine dye, $\text{C}_{56}\text{H}_{68}\text{Cl}_4\text{CuN}_{16}\text{S}_4$.

Absorption characteristics

Dissolve 35 mg of the dye in 100 ml water, then dilute 5 ml of this solution to 50 ml with water. Read in a spectrophotometer in a 1 cm cell.

Absorption maximum, 611–620 nm; P–15/P+15 ratio, between 1.09 and 1.21.

Spectrophotometric assay

Percentage of dye = D peak $\times$ 67.2. D-peak (color density at peak) is measured for the dye solution described above. For certification, samples of this dye should contain $\geq$ 50% dye.

Biological tests

Alcian blue is tested on paraffin sections of small intestine, trachea and larynx using both Scott's and Mowry's methods, and also as a component of Kreyberg's method for keratin and mucus.

Scott's alcian blue method (Scott and Dorling 1965). In this method, alcian blue is used at pH 5.7 with 0.3 M magnesium chloride, for staining polysulfates. The alcian blue-magnesium chloride staining solution is made by dissolving 24 mg alcian blue in a mixture consisting of 2.4 ml 0.5 M acetic acid-sodium acetate buffer, pH 5.7, 3.6 ml 4.0 M MgCl_2 and 42 ml water. The final dye solution should be clear and remain so for at least 24 h. Alcian blue 8GX that fails to yield a clear solution may have deteriorated and should be replaced by one that is more soluble.

Take paraffin sections through xylenes and alcohol, rinse briefly in water, and stain overnight (18–24 h) in the alcian blue-magnesium chloride staining solution. Rinse in three changes of deionized water. At this point, sections can be counterstained for 5 min with nuclear fast red (see Mowry's method below) if desired, then washed in water. The stained sections are dehydrated in 95% and absolute ethanol, cleared in xylene and mounted in a synthetic resin.

Polyanions rich in sulfate esters (cartilage matrix, mast cell granules, some types of epithelial mucus) are colored turquoise-blue whereas polycarboxylates devoid of sulfates are unstained. The cytoplasm of cells generally either remain unstained or are only slightly colored. Nuclei are pink with the suggested counterstain.

Mowry's alcian blue (pH 2.5) method (McManus and Mowry 1960). In this method, alcian blue is used at approximately pH 2.5 to stain both sulfated and nonsulfated acidic mucosubstances.

Deparaffinize and hydrate sections. Place in 3% acetic acid for 3 min. Stain in the alcian blue solution (alcian blue 8GX, 0.5 g; 3% acetic acid, 100 ml) for 30 min. Wash in running tap water for 1 min. Stain in nuclear fast red solution (nuclear fast red, 0.1 g; 5% aluminum sulfate ($\text{Al}_2(\text{SO}_4)_3 \cdot 18\text{H}_2\text{O}$) solution, 100 ml; dissolve with heat) for 3 min. Rinse in water, dehydrate, clear and mount.

Acidic mucosubstances stain blue, nuclei red.

Kreyberg's method. Kreyberg's method is described later in connection with phloxine B. Acidic mucosubstances appear blue as with Mowry's method. Nuclei are black and other components of the tissue are pink to red.

Alizarin red S (C.I. 58005)

Alizarin red S is the sodium salt of 1,2-dihydroxyanthraquinone-3-sulfonic acid, $\text{C}_{14}\text{H}_7\text{O}_7\text{SNa}$, with one molecule of water of crystallization.

Absorption characteristics

Dissolve 50 mg of alizarin red S in 250 ml 0.1 N NaOH. Dilute 15 ml of this solution to 200 ml with 0.1 N NaOH. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 593–599 nm; P–15/P+15 ratio; between 0.98 and 1.07 for the first maximum.

Assay

The BSC does not assay this dye.

Biological test

Alizarin red S is tested using Dahl's method for calcium (Dahl 1952, Churukian 2000a). Deparaffinize sections of any formaldehyde fixed specimen known to contain calcified deposits, and bring to 95% alcohol. Drain and allow sections to dry. Stain in alizarin red S solution (alizarin red S, 1 g; water, 100 ml; adjust to pH 7.0 with 1% ammonium hydroxide) for 5 min. Rinse in water and counterstain with fast green FCF solution (fast green FCF, 0.2 g; water, 100 ml; acetic acid, 0.2 ml; dilute 10 ml of this stock solution with 35 ml water) for 40 sec. Rinse in water, dehydrate with alcohol, clear in xylene and mount.

Calcium salts stain reddish orange against a pale green background.

Aniline blue (C.I. 42780)

Aniline blue consists of sulfonation products of variable mixtures of phenylated rosaniline and pararosaniline. These include methyl blue (C.I. 42780; acid blue 93) and water blue (C.I. 42755; acid blue 22). Presently, methyl blue is the predominant product (see Chapter 14 in Horobin and Kiernan 2002), but dyes sold as methyl blue are also likely to be mixtures.

Absorption characteristics

Dissolve 50 mg aniline blue in 250 ml of water. Dilute 10 ml of this solution and 10 ml 0.1 N HCl to

200 ml with water. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 594–610 nm; P–15/P+15 ratio, between 0.96 and 1.09.

Assay

Dissolve 200 mg dye in 175 ml water, add 25 ml of pH 4.5 acetate buffer, heat to boiling, and titrate with 0.05 N TiCl_3 . The end point will vary from yellow to dark yellow-green. The BSC specifies that the minimum acceptable TiCl_3 consumption per gram of dye is 12.0 ml of 0.1 N TiCl_3 solution. The variable nature of aniline blue precludes correlation of this standard with a percentage by weight of dye in the original sample.

Decolorization test

Add 2 ml of 1.0 N NaOH to 100 ml of a 0.01% solution of the dye. The color should turn red immediately, fade after 10 min to a straw color, and become almost colorless after 20 min. Drop-wise addition of 1.0 N HCl should restore the blue color almost instantly.

Biological test

Aniline blue is tested in Mallory's connective tissue stain on animal tissue, such as kidney or intestine, after fixation in Bouin's, Zenker's or Carnoy's fluid, and embedment in celloidin or paraffin. The procedure used is given under acid fuchsin above.

With a good sample of aniline blue, collagen fibrils, reticulum, amyloid, and mucus stain blue. Aniline blue is also tested in Gomori's one-step trichrome stain, as an alternative to fast green FCF (see fast green FCF below).

Auramine O (C.I. 41000)

Auramine O has the empirical formula $\text{C}_{17}\text{H}_{22}\text{N}_3\text{Cl}$.

Absorption characteristics

Dissolve 50 mg auramine O in 250 ml water. Dilute 5 ml of this solution to 200 ml with water. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 431–433 nm; P–15/P+15 ratio, between 1.02 and 1.10.

Spectrophotometric assay

The percentage of dye = $D_{\text{peak}} \times 148$. D_{peak} (color density at peak) is measured for the dye solution described above. Samples must contain $\geq 80\%$ dye.

Biological test

Auramine O is tested by fluorescence microscopy

using Churukian's modification (Churukian 1991) of Truant's fluorescent method for acid-fast bacilli on paraffin sections of formaldehyde fixed lung known to contain tuberculosis lesions.

The staining solution contains auramine O, 0.45 g; rhodamine B (C.I. 45170), 0.03 g; glycerol, 90 ml; water, 60 ml; ethanol, 12 ml; 90% liquid phenol, 13.2 ml. Combine the liquids, add the dyes, place on a magnetic stirrer over gentle heat, and mix for 30 min.

Deparaffinize slides and hydrate. Place in the auramine O-rhodamine B solution and put in a 600 watt microwave oven at power level 1 (60 W) for 3 min. Agitate for 15 sec, and leave in the warm solution for 2 min. An alternative method is to stain the slides for 15 min at room temperature. Wash the slides with water and differentiate in two changes of acid-alcohol (0.5% concentrated HCl in 70% ethanol), 1 min in each change. Place in an eriochrome black T solution (eriochrome black T, C.I. 14645, 0.3 g; water, 100 ml) for 10 sec. Rinse in water, air dry, dip in xylene and mount.

Acid-fast bacteria should fluoresce orange-yellow or yellow. No other fluorescent colors are seen in the sections. (Absorption maxima are 434 nm for auramine O and 543 nm for rhodamine B. Emission maxima are 550 for auramine O and 585 for rhodamine B.) Heating the auramine O solution to 70° C in a microwave oven causes the acid-fast bacteria to fluoresce a brighter yellow than when staining is carried out at room temperature.

Azocarmine G (C.I. 50085) and azocarmine B (C.I. 50090)

These dyes are the mono- and disodium salts of the di- and trisulfonic acid derivatives of azocarmine base: azocarmine G, $\text{C}_{28}\text{H}_{18}\text{N}_3\text{O}_6\text{S}_2\text{Na}$; azocarmine B, $\text{C}_{28}\text{H}_{17}\text{N}_3\text{O}_9\text{S}_3\text{Na}_2$.

Absorption characteristics

Dissolve 50 mg azocarmine G or B in 250 ml water. Dilute 20 ml of this solution to 200 ml with water. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 510–514 nm; P–15/P+15 ratio, between 0.92 and 0.96.

Assay

Dissolve 200 mg dye in 200 ml water. Add 10 g sodium bitartrate ($\text{NaHC}_4\text{H}_4\text{O}_6 \cdot \text{H}_2\text{O}$), heat to boiling, and titrate with 0.05 N TiCl_3 to an orange end point. The following data are used to calculate the percentage of dye in the sample.

	Azocarmine G	Azocarmine B
Molecular weight	579.590	581.634
Hydrogen equivalents per mole of dye	2	2
Milliliters of N/10 TiCl ₃ per gram of dye	34.507	29.341

Dye contents of satisfactory samples are commonly 75–80% for azocarmine G and 80–85% for azocarmine B.

Biological test

The two dyes are tested as the red component of Heidenhain's AZAN trichrome stain on paraffin sections of kidney and small intestine fixed in Zenker's or Bouin's fluids. Sections of formaldehyde fixed tissues may be rendered amenable to staining by pretreatment with picric acid. The procedure is explained in more detail by Gabe (1976) and Kiernan (1999). Azocarmine B, which is the more soluble dye, can be used in higher concentrations than azocarmine G and has been preferred by many workers.

Add 1 g of either dye to 100 ml of water, heat to boiling, cool to room temperature and filter. Acidify cooled filtrate by adding 1 ml glacial acetic acid. Do not filter the acidified solution even though at room temperature it may contain undissolved material. Heat the azocarmine solution to 55° C before using.

The paraffin is removed and the sections are hydrated in the usual manner. If the fixative contained mercuric chloride (e.g., Zenker's fluid) the slides are treated with Gram's iodine (1% I₂ in 2% aqueous KI) followed by 5% aqueous sodium thiosulfate (Na₂S₂O₃·5H₂O) to remove mercury deposits. If the fixative did not contain HgCl₂ or picric acid, sections are immersed in a saturated aqueous solution of picric acid for 60 min at 50–60° C or overnight at room temperature. No pretreatment is needed after fixation in Bouin's.

Sections are washed in two changes of water before staining. Slides are immersed in the pre-heated azocarmine solution for 60 min in a covered dish at 55° C (the temperature must not be allowed to rise above 60° C), then agitated in running tap water for a few sec to remove particles of undissolved dye, and passed through 2 changes of pure water. The sections are differentiated one at a time in 0.1% aniline in 95% alcohol until red nuclei stand out prominently. This usually takes 2 or 3 min (range 30 sec to 10 min). The differentiation is stopped by rinsing in acetic-alcohol (1% acetic acid in 95% alcohol), and the slides are moved into 5% aqueous phosphotungstic acid until all pink color

has been removed from collagen, which usually takes 30–60 min, but occasionally as long as 3 h. The sections then are rinsed in slightly acidified water (0.5% acetic acid) and stained for 1–3 h in a 1:2 aqueous dilution of a stock solution consisting of certified aniline blue, 0.5 g; certified orange G, 2 g; glacial acetic acid, 8 ml; and water, 100 ml. Finally, the sections are rinsed in slightly acidified water, dehydrated in 3 changes of absolute alcohol, cleared in xylene, and mounted.

A satisfactory AZAN stain produces bright red nuclei and erythrocytes, orange muscle, blue mucus and cartilage, and dark blue reticular fibers and collagen. Certain cell types may have pink, red, yellow or blue cytoplasm.

Azure A (C.I. 52005)

Azure A is 3-amino-7-dimethylamino-3-iminophenothiazine hydrochloride (asymmetric dimethylthionine), C₁₄H₁₄N₃SCl.

Absorption characteristics

Dissolve 50 mg azure A in 250 ml water. Dilute 3 ml of this solution to 200 ml with water. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 628–634 nm; P–15/P+15 ratio, between 0.94 and 1.20.

Chromatography

TLC is performed to examine the composition of the dye (Fig. 2). HPLC is also performed to examine the purity of the dye sample. At least 33% of the total dye content should be azure A.

Assay

Dissolve 100 mg dye in 175 ml of water, add 25 ml pH 4.0 acetate buffer (6 volumes of 50% NaOOCCH₃·3H₂O + 4 volumes of glacial acetic

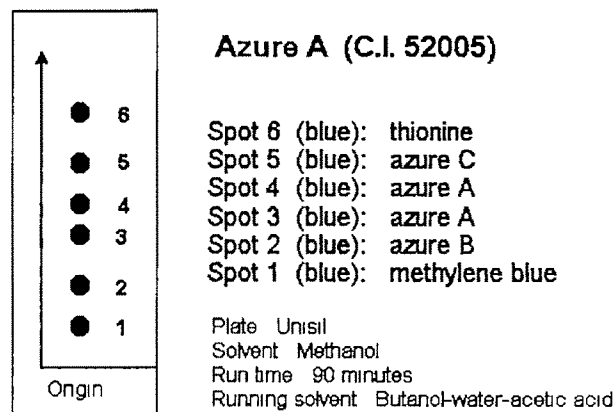


Fig. 2. TLC of a sample of azure A.

acid), heat to boiling, and titrate with 0.05 N TiCl_3 to a yellow end point. The following data are used to calculate the percentage of dye in the original sample.

Molecular weight	291.813
Hydrogen equivalents per mole of dye	2
Milliliters of N/10 TiCl_3 per gram of dye	68.537

For certification, samples of this stain must contain $\geq 75\%$ titratable dye.

Biological tests

Azure A is tested in techniques applied to paraffin sections and to blood smears.

Lillie's modified Nocht's method (Lillie and Fullmer 1976). Azure A is tested in Lillie's modified Nocht's method on Zenker or formalin fixed bone marrow. This technique is also used to test azure B, azure C, eosin B, eosin Y, toluidine blue O and the blood stains of Wright and Jenner. All dyes except the one being tested must be from batches previously certified by the BSC.

Bring paraffin sections to water in the usual manner using a 0.5% iodine, 5% sodium thiosulfate sequence for mercuric chloride fixed material. Stain for 1 h in a Coplin jar containing: 0.1% aqueous azure A (or B or C or toluidine blue O), 4 ml; 0.1% aqueous eosin B (or eosin Y), 4 ml; acetone, 5 ml; water, 25 ml; and buffered at pH 4.1 with 0.1M citric acid, 1.2 ml; and 0.2 M disodium hydrogen phosphate, 0.8 ml. For making up Nocht-type solutions from blood stains, see Wright's and Jenner's stains below.

Dehydrate the stained slides in two or three changes of acetone, clear in a 1:1 acetone:xylene mixture followed by two changes of xylene, and mount in a synthetic resin. With good samples of azure A and eosin B, nuclei are blue, mast cell granules blue-violet, cartilage matrix reddish violet, cytoplasm light blue to violet or lavender, muscle fibers bright pink, and erythrocytes orange-red.

NCCLS method for blood smears. This technique is used for testing azure A, azure B and azure C. Prepare stock methanolic solutions of azure A (or azure B or azure C) (260 mg/100 ml methanol) and of certified eosin Y (130 mg/100 ml methanol).

Stain methanol fixed blood smears by immersing them in a mixture of azure A (or B or C) solution (1 volume), eosin Y solution (1 volume) and 0.067 M Sorensen's sodium phosphate buffer, pH 6.8 (10 volumes) for 10 min. The solution must be used within 1 h of preparation. The stained smears are rinsed in water and air dried. For a permanent

mount, slides are dipped in xylene and mounted in a synthetic resin.

The colors are similar to those seen with other Romanowsky-Giemsa stains (see Chapter 21 in Horobin and Kiernan 2002).

Azure B (C.I. 52010)

Azure B is trimethyldiaminodiphenothiazine hydrochloride (trimethylthionine), $\text{C}_{15}\text{H}_{16}\text{N}_3\text{S}\text{Cl}$.

Absorption characteristics

Dissolve 50 mg azure B in 250 ml water. Dilute 3 ml of this solution to 200 ml with water. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 645–649 nm; P–15/P+15 ratio, between 1.02 and 1.18.

Chromatography

TLC is performed to examine the composition of the dye (Fig. 3). HPLC is performed to test purity of the dye sample, which consists mostly of azure B.

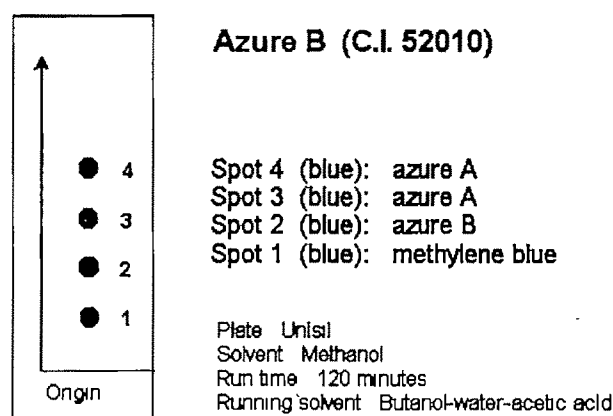


Fig. 3. TLC of a sample of azure B.

Assay

Dissolve 100 mg dye in 175 ml water, add 25 ml of pH 4.0 acetate buffer (6 volumes of 50% $\text{NaOOCCH}_3 \cdot 3\text{H}_2\text{O}$ + 4 volumes of glacial acetic acid), heat to boiling, and titrate with 0.05 N TiCl_3 to a yellowish gray end point. The following data are used to calculate the percentage of dye in the original sample

Molecular weight	305.840
Hydrogen equivalents per mole of dye	2
Milliliters of N/10 TiCl_3 per gram of dye	65.394

For certification, samples of this dye should contain $\geq 89\%$ dye.

Biological test

Azure B is tested in the same way as azure A. Azure B is the only dye that provides the classical Romanowsky-Giemsa coloration of blood cells (see Chapter 21 in Horobin and Kiernan 2002).

Azure C (C.I. 52002)

Azure C is 3-amino-7-methylaminophenothiazine hydrochloride (monomethyl thionine), $C_{13}H_{12}N_3SCl$.

Absorption characteristics

Dissolve 50 mg azure C in 250 ml water. Dilute 3 ml of this solution to 200 ml with water. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 608–622 nm; P–15/P+15 ratio, between 0.93 and 1.03.

Chromatography

TLC is performed to examine the composition of the dye (Fig. 4). HPLC is performed to test the purity of the dye sample.

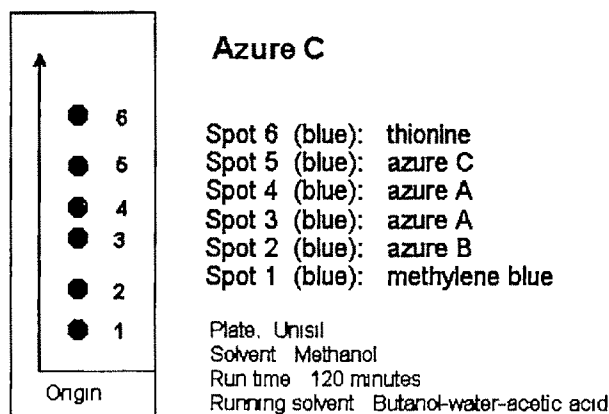


Fig. 4. TLC of a sample of azure C.

Assay

Dissolve 100 mg dye in 175 ml water, add 25 ml of pH 4.0 acetate buffer (6 volumes of 50% $NaAc \cdot 3H_2O$ + 4 volumes of glacial acetic acid), heat to boiling, and titrate with 0.05 N $TiCl_3$ to a yellow end point. The following data are used to calculate the percentage of dye in the original sample.

Molecular weight	277.786
Hydrogen equivalents per mole of dye	2
Milliliters of N/10 $TiCl_3$ per gram of dye	71.998

For certification, samples of this stain should contain $\geq 70\%$ dye.

Biological test

Azure C is tested in the same way as azure A.

Basic fuchsin

Basic fuchsin is commonly a mixture of pararosaniline (C.I. 42500), rosaniline (C.I. 42510), magenta II (no CI number) and new fuchsin (CI 42520). For reasons explained by Horobin and Kiernan (2002, Chapter 14), pararosaniline and new fuchsin are the only components of basic fuchsin available as single dyes.

Rosaniline, also known as magenta I, is triaminotolylidiphenylmethane chloride, $C_{20}H_{20}N_3Cl$. It is also sold as an acetate, $C_{30}H_{20}N_3C_2H_3O_2$. Pararosaniline also may be a chloride or acetate salt. Although both dyes are marketed under the general name of basic fuchsin, each should be indicated on the label by the appropriate subtitle and corresponding C.I. number.

Pure pararosaniline is commercially available and its use is mandatory for some techniques. This dye, therefore, is tested and certified separately from samples sold as basic fuchsin (see pararosaniline below).

Absorption characteristics

Dissolve 50 mg basic fuchsin in 125 ml 95% alcohol, shake for 20 min, then dilute to 250 ml with water. Dilute 3 ml of this solution to 200 ml with 50% ethyl alcohol. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 547–550 nm; P–15/P+15 ratio, between 1.16 and 1.35.

Test for acetate

To test for the acetate ion, add 1 ml 6 N H_2SO_4 to 2.0 g dye dissolved in 5 ml of water and heat. The

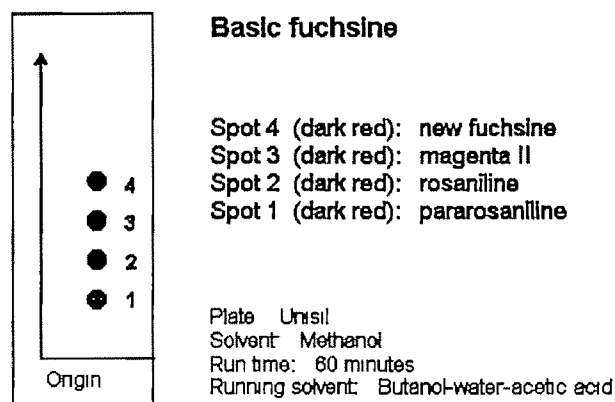


Fig. 5. TLC of a sample of basic fuchsin.

vinegar-like odor of acetic acid escaping from the hot mixture indicates the presence of acetate in the original sample. If acetate is not present, the sample is assumed to be the chloride.

Chromatography

TLC is performed to determine the composition of the dye mixture (Fig. 5). The approximate percentage of each component in the sample is determined by HPLC, which usually reveals approximately equal proportions of rosaniline and pararosaniline in samples of basic fuchsin that are mixtures of dyes. An average molecular weight, therefore, is used.

Assay

Dissolve 100 mg dye in 175 ml of water, add 25 ml 30% sodium tartrate ($\text{Na}_2\text{C}_4\text{H}_4\text{O}_6 \cdot 2\text{H}_2\text{O}$), heat to boiling, and titrate with 0.05 N TiCl_3 to a yellow end point. The following data are used to calculate the percentage of dye in the sample.

Rosaniline	Chloride	Acetate
Molecular weight	337.861	361.450
Hydrogen equivalents per mole of dye	2	2
Milliliters of N/10 TiCl_3 per gram of dye	59.196	55.332
Pararosaniline	Chloride	Acetate
Molecular weight	323.834	347.423
Hydrogen equivalents per mole of dye	2	2
Milliliters of N/10 TiCl_3 per gram of dye	61.760	57.566
Rosaniline & pararosaniline mixture	Chloride	Acetate
Molecular weight	330.9	354.5
Hydrogen equivalents per mole of dye	2	2
Milliliters of N/10 TiCl_3 per gram of dye	60.5	56.5

New fuchsin and magenta II, are not allowed for in these calculations, but any error due to their presence in a mixture must be small because the dyes of the basic fuchsin group do not differ greatly in their molecular weights. New fuchsin is commercially available (see Chapter 14 in Horobin and Kiernan 2002), but it is not currently among the dyes certified by the BSC. For certification, samples of basic fuchsin should contain $\geq 88\%$ dye.

Decolorization test

Dissolve 0.5 g dye in 100 ml boiling water, cool to 50°C , filter into a small flask, and add 10 ml 1.0 N

hydrochloric acid to the filtrate. Add 0.5 g potassium metabisulfite, $\text{K}_2\text{S}_2\text{O}_5$, shake until dissolved, stopper tightly and allow to stand in the dark for 12–18 h. The solution is a yellowish orange. A yellowish brown or brown solution, especially in the presence of a dark sediment, indicates a poorer grade of fuchsin, but a sample that fails this test is not rejected if it meets the other requirements for certification.

Biological tests

Basic fuchsin is tested for several purposes: 1) as a stain for the tubercle bacillus, 2) in the Endo medium for distinguishing between the bacteria *Escherichia coli* and *Aerobacter aerogenes*, 3) in the periodic acid-Schiff (PAS) method, 4) in the Feulgen stain, and 5) some batches are tested for staining bacterial flagella.

Acid-fast staining with carbol-fuchsin. Carbol-fuchsin is a solution containing basic fuchsin and phenol. It is used to stain tubercle bacilli (*Mycobacterium tuberculosis*) and other organisms that retain the dye after immersion in acidified 70% alcohol. The classical Ziehl-Neelsen technique is for detecting tubercle bacilli in smears. The BSC Laboratory performs the Ziehl-Neelsen and Kinyoun's (Churukian 2000a) variant techniques on paraffin sections of a formaldehyde-fixed lung that is known to contain tubercle bacilli. Instead, Kinyoun's variant (Churukian 2000a) is applied to paraffin sections of a formaldehyde fixed lung that is known to contain tubercle bacilli.

The carbol-fuchsin for use on sections is prepared by dissolving 4 g basic fuchsin and 8 g phenol in 20 ml 95% alcohol by heating, followed by addition of 100 ml water.

Stain deparaffinized and hydrated sections in the carbol-fuchsin solution for 1 h, rinse in tap water, and differentiate in 70% alcohol containing 1% hydrochloric acid until the section is light pink. After washing in tap water, counterstain with acidified 0.1% aqueous methylene blue for 1 min. Dehydrate the preparation in alcohol, clear in xylene, and mount in a resinous medium. The tubercle bacilli should be red and the nuclei of cells blue.

Endo medium. To test for use in the Endo medium, two solutions are prepared of the sample of basic fuchsin to be tested: 1% alcoholic and 3% alcoholic. Of these solutions, 1 ml of each is mixed separately with 0.125 g sodium sulfite dissolved in 5 ml hot water. The tests are carried out separately with each solution; good results must be obtained with at least

one of them. The solution should be a faint pink or straw color, or sometimes light brown, but without noticeable precipitate. This decolorized solution is added to 100 ml melted lactose agar of the standard formula. The color of this medium should be a light pink, which fades almost entirely on cooling. Before cooling, it is poured into a Petri dish and allowed to harden. The surface is then streaked in parallel lines with *Escherichia coli* and *Aerobacter aerogenes* and incubated for 24 h at 37° C.

A good sample should show red growth with a strong metallic sheen for the former organism, pink growth without a metallic sheen for the latter, and no reddening of the medium except where growth has occurred.

Schiff's reagent. Schiff's reagent, which produces a colored product with aldehydes, is used in the periodic acid-Schiff and Feulgen techniques. The reagent is sometimes called leucofuchsin, but this is not a satisfactory term (see Chapter 14 in Horobin and Kiernan 2002). It is prepared from the following ingredients combined in the order listed.

Water	80 ml
Basic fuchsin	1 g
Either sodium bisulfite (NaHSO ₃) or	2 g
Sodium metabisulfite (Na ₂ S ₂ O ₅)	1.9 g
1.0 N hydrochloric acid	20 ml

Shake at intervals for 2 h, then add 500 mg finely powdered fresh charcoal. Shake the mixture for 1 min and filter. The filtrate should be clear and colorless. It is stored in a securely capped bottle.

Periodic acid-Schiff (PAS) method. This is a histochemical stain for neutral polysaccharides, neutral glycoproteins and some sialoglycoproteins. For a more detailed account of the method used, see Churukian (2000a).

Specimens containing neutral mucosubstances (bowel, kidney and liver are suitable) are fixed in formaldehyde, Zenker's or Bouin's fluid. Paraffin sections are dewaxed and hydrated in the usual way, then rinsed in tap water, oxidized for 10 min in 0.5% aqueous periodic acid, washed for 5 min in running tap water, and immersed for 15 min in Schiff's reagent. The sections are rinsed in three changes of water, followed by a 15 sec rinse in a 0.3% aqueous solution of borax, then four more rinses in water. Nuclei are counterstained with a progressive alum-hematoxylin (such as Mayer's). The stained slides are washed in tap water, dehydrated through graded alcohols, cleared in xylene and mounted in a synthetic resin.

A satisfactory stain should show collagen pink to red, basement membranes purplish red, epithelial

mucus red-purple to violet, glycogen dark purplish red, nuclei blue, and cytoplasm gray. In plant tissue, cellulose is red and starch is dark purple.

Feulgen technique (Churukian 2000a). This is a specific histochemical stain for DNA. The treatment with hydrochloric acid causes hydrolysis of nucleic acids. RNA is destroyed and extracted from the tissue, but DNA is converted to a polyaldehyde.

Onion root tips are fixed in CRAF (10% aqueous chromium trioxide, 3 ml; 10% acetic acid, 20 ml; formalin (37–40% HCHO), 10 ml; water, 67 ml) and intestine is fixed in Zenker's fluid. Paraffin sections of these objects are dewaxed and brought to water in the usual manner. The sections are immersed in 5 N hydrochloric acid (42 ml concentrated acid added to 58 ml water) at room temperature for 30 min, then transferred to Schiff's reagent for 30 min. The slides are then rinsed in three changes of water, followed by immersion for 15 sec in 0.3% borax and four more rinses in water. The Feulgen-stained sections are counterstained for 10 sec with a working solution of fast green FCF made by adding 10 ml of a stock solution (0.2% fast green FCF in 0.2% aqueous acetic acid) to 35 ml water. The slides then are dehydrated through graded alcohols, cleared in xylene and mounted in a synthetic resin.

With a satisfactory sample of basic fuchsin, cell nuclei (the main location of DNA) are stained magenta by the Feulgen method. Other tissue elements are counterstained in shades of green. Elastin (of young animals) and lignin are also colored red, but this staining, due to pre-existing aldehyde groups, occurs also if hydrochloric acid hydrolysis is omitted.

"Special" basic fuchsin for flagellar staining. Samples sold under this designation are presumed to be 3:1 (w/w) mixtures of pararosaniline acetate and chloride as suggested by Leifson (1951). Basic fuchsin certified as "special for flagella" is tested only by this method. It does not have to pass any other tests, and is therefore not necessarily suitable for making carbol-fuchsin or Schiff's reagent. Any organism with flagella may be used as test material. It is convenient to use prepared slides from a commercial supplier. The organism currently used is *Curthidia*.

Leifson's flagella stain. The staining solution for bacterial flagella is made by combining equal volumes of 1.5% NaCl in water, 3% tannic acid in water, and a 1.2% solution of the basic fuchsin dye sample in 95% alcohol.

Place a loopful of sediment from a centrifuged 24 h broth culture of motile bacteria at the end of a

clean glass slide and tilt, causing liquid to flow and spread. Air dry, then quickly add 1 ml of stain. After 5–15 min at room temperature, rinse the slide with tap water under a faucet, but without pouring the stain off before rinsing. Drain dry.

A satisfactory sample of basic fuchsin should stain the flagella bright red.

Bismarck brown Y (C. I. 21000)

Bismarck brown Y is the dihydrochloride of *m*-phenylenebis(azo)*bis-m*-phenylenediamine, $C_{18}H_{20}N_8Cl_2$.

Absorption characteristics

Dissolve 50 mg Bismarck brown Y in 125 ml 95% alcohol, then dilute to 250 ml with water. Dilute 10 ml of this solution and 1 ml 1.0 N HCl to 200 ml with 50% alcohol. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 454–458 nm; P–15/P+15 ratio, between 0.97 and 1.08.

Assay

Dissolve 100 mg dye in 100 ml alcohol and 100 ml water, add about 10 g sodium bitartrate ($NaH-C_4H_4O_6 \cdot H_2O$), heat to boiling, and titrate with 0.05 N $TiCl_3$. The end point, which is not always sharp, ranges from pale yellow to brownish yellow. The following data are used to calculate the percentage of dye.

Molecular weight	419.336
Hydrogen equivalents per mole of dye	8
Milliliters of N/10 $TiCl_3$ per gram of dye	190.778

For certification, samples of this dye must contain $\geq$ 45% dye.

Biological test

Bismarck brown Y is tested on paraffin sections of Zenker fixed intestine and trachea. The sections are brought to water, stained in a 1% aqueous solution of Bismarck brown Y for 5–10 min, rinsed in 95% alcohol, dehydrated, cleared and mounted. Satisfactory staining shows light brown mucus (goblet cells) and deep brown cartilage. (Counterstains do not work well.)

Brilliant cresyl blue (C.I. 51010)

In general, samples of this stain are aminodimethylaminomethyldiphenoxazonium chlorides, $C_{17}H_{20}N_3OCl$ (M.W. 317.818), not the compound illustrated in the *Colour Index* (C.I. 51010), which is a diaminomethyldiethylaminodephenoxyazonium chloride (M.W. 289.774). The latter may never have

been commercially available (Green 1990, 2000). See Chapter 18 in Horobin and Kiernan (2002) for more information about this dye.

Absorption characteristics

Dissolve 50 mg brilliant cresyl blue in 125 ml water, then dilute to 250 ml with 95% alcohol. Dilute 5 ml of this solution to 200 ml with 50% alcohol. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 624–628 nm; P–15/P+15 ratio, between 1.09 and 1.30.

Assay

Dissolve 100 mg dye in 100 ml 95% alcohol, add 100 ml water and 10 g sodium bitartrate ($NaH-C_4H_4O_6 \cdot H_2O$), heat to boiling, and titrate with 0.05 N $TiCl_3$ to the first appearance of a lighter reddish brown color. The following data are used to calculate the percentage of dye.

Molecular weight	289.774
Hydrogen equivalents per mole of dye	2
Milliliters of N/10 $TiCl_3$ per gram of dye	66.717

For certification, samples of this stain must contain $\geq$ 60% dye, calculated as if it were the *Colour Index* (C.I. 51010) compound.

Note. A more correct assay, based on the actual structure of the dye usually tested, would use the following data:

Molecular weight	317.82
Hydrogen equivalents per mole of dye	2
Milliliters of N/10 $TiCl_3$ per gram of dye	60.83

Biological tests: supravital staining methods for reticulocytes. Brilliant cresyl blue is tested by two methods for staining reticulocytes in blood. In normal human blood, 1–2% of the red cells are reticulocytes stainable with brilliant cresyl blue.

1. Fresh blood is examined under a coverglass on which a thin film of a filtered 0.3% solution of brilliant cresyl blue in ethyl or methyl alcohol has been dried. Alternatively, blood films are made on coverglasses, dried, and counterstained with Wright's stain.

The reticulum of immature red cells should be a deep blue on a very pale blue (fresh) or eosin colored (counterstained) background. Blood platelets should stain pale blue or lilac and should be discrete, with only a minimum of precipitate or debris.

2. An aqueous solution of brilliant cresyl blue (1% in 1.5% potassium oxalate) is also tested using a supravital wet preparation. Three drops of the dye solution are mixed on a slide with 3 drops of anticoagulated blood. One drop of the mixture then

is transferred to another slide, covered, and examined.

Reticulocytes should be stained deep blue as above.

Brilliant green (C.I. 42040)

Brilliant green is the acid sulfate of a basic triphenylmethane dye, $C_{27}H_{33}N_2SO_4H$.

Absorption characteristics

Dissolve 50 mg brilliant green in 250 ml 50% alcohol. Dilute 3 ml of this solution to 200 ml with 50% alcohol. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 626–630 nm; P–15/P+15 ratio, between 0.94 and 1.14.

Assay

Dissolve 100 mg dye in 100 ml 95% alcohol. Add 100 ml water and 10 g sodium bitartrate ($NaHC_4H_4O_6 \cdot H_2O$). Heat to boiling, and titrate with 0.05 N $TiCl_3$ to a pale yellow end point. The following data are used to calculate the percentage of dye.

Molecular weight	482.651
Hydrogen equivalents per mole of dye	2
Milliliters of N/10 $TiCl_3$ per gram of dye	41.438

For certification, samples must contain $\geq 90\%$ dye.

Biological test

Brilliant green is tested as a bacteriostatic agent. Make four daily transfers at $37^\circ C$ of a spore-former (e.g., *Bacillus cereus*) and two members of the colony-typoid group (*Enterobacter aerogenes* and *Escherichia coli*) into a broth containing 1% peptone and 1% lactose. As soon as distinct turbidity appears in the fourth transfer (3–6 h incubation), count the organisms under a microscope using a hemocytometer (it is desirable to have the count between 4 and 20 million/ml). Dilute with water so that the concentration of bacteria is approximately 200/ml.

Meanwhile, make a 0.01% aqueous solution of each sample of brilliant green to be tested and of a control (already certified) sample. To make these solutions, add 10 mg dye to a 100 ml volumetric flask not quite full of water; bring up to the volume mark and allow to stand overnight. From these flasks prepare 0.001, 0.0001 and 0.00001% solutions.

Fill a series of test tubes with 7.5 ml of a broth containing 1.33% peptone and 1.33% lactose, and divide into three lots, one for each of the three test organisms. To each of these three series of tubes add varying quantities of the dye by introducing with a pipette 0.2–2.0 ml of the four dilutions mentioned

above, then bring the total volume in each tube up to 9.5 ml by adding water and mixing thoroughly. In this way final dilutions from 1:50,000 to 1:500,000,000 may be obtained, although partial sets can be selected for each organism, e.g., from 1:5,000,000 to 1:100,000,000 for the spore-former and from 1:50,000 to 1:2,000,000 for the others.

Sterilize the tubes in an autoclave and cool to room temperature. Inoculate each set of the tubes containing broth and diluted dye with 0.5 ml of the diluted culture (i.e., approximately 100 organisms) per tube. Mix each tube well and incubate at $37^\circ C$. Examine for growth on the 1st, 2nd and 4th days. A satisfactory sample of brilliant green should show a bacteriostatic effect on all three organisms sufficiently similar to the control sample that the first dilution of dye that permits growth is no more than two stages from the control in the series of final dilutions above.

Carmines (C.I. 75470)

Carmines is a cochineal product usually furnished as an aluminum and calcium salt of carminic acid, $C_{22}H_{20}O_{13}$. The composition of carmines is discussed in Chapter 25 of Horobin and Kiernan (2002).

Absorption characteristics

The BSC does not test carmines spectrophotometrically.

Assay

Spectrophotometric assays are available for carmines and carminic acid (Marshall and Horobin 1974a), but no attempt is made to determine the dye content in samples of carmines submitted for certification.

Biological tests

Carmines is tested in Orth's lithium carmines technique for nuclear staining, Southgates's mucicarmines method and Best's stain for glycogen.

Orth's lithium carmines technique. Paraffin sections of animal tissue are prepared, following any fixative. The paraffin is removed in the usual manner and the sections are stained 2–5 min in a carmines solution. To prepare carmines stain, 2.5–5 g carmines is dissolved in 100 ml saturated aqueous lithium carbonate (approx. 1.25%), boiled for 10–15 min, cooled, about 0.1 g thymol added, then filtered. Transfer stained slides directly to acid-alcohol (1% conc. HCl in 70% alcohol), one or more changes for several min. Wash in tap water, dehydrate, clear and mount.

A satisfactory sample of carmine should produce red nuclei and the cytoplasm should be light pink or unstained.

Southgate's mucicarmine method. Prepare a mucicarmine stock solution: add 1 g carmine to 100 ml 50% alcohol, mix and add 1 g aluminum chloride hexahydrate and 1 g aluminum hydroxide. Heat and boil for 3 min. Restore the volume with 50% alcohol and filter through glass wool.

Deparaffinize sections of small intestine and take to water. Stain nuclei with Weigert's hematoxylin (see hematoxylin below), wash in running tap water, and stain in a working mucicarmine solution (stock solution diluted 1:3 with water) for 1.5–3 h until mucus in goblet cell is adequately stained. Rinse in water and counterstain with tartrazine (tartrazine [C.I. 19140], 0.1 g; water, 100 ml; acetic acid, 0.2 ml) for 10 sec, rinse in water, dehydrate, clear and mount.

Mucus stains rose to red, nuclei black, and background yellow-orange.

Best's carmine for glycogen. Prepare a stock solution of carmine: combine 2 g carmine, 1 g potassium carbonate, 5 g potassium chloride and 60 ml water. Boil gently for 5 min. Cool and add 20 ml 28% ammonium hydroxide.

Deparaffinize and hydrate sections of liver (preferably following a nonaqueous fixative). Stain nuclei in Weigert's hematoxylin (see hematoxylin below), and rinse in water. Stain in a working carmine solution (carmine stock solution, 10 ml; 28% ammonium hydroxide, 15 ml; methyl alcohol, 15 ml) for 30 min. Rinse in differentiating solution (absolute alcohol, 20 ml; methyl alcohol, 10 ml; water, 25 ml) for 3 sec. Rinse in 70% alcohol, dehydrate, clear and mount.

Glycogen is stained pink to red.

Chlorazol black E (C. I. 30235)

Chlorazol black E has the empirical formula $C_{34}H_{25}N_9O_7S_2Na_2$ with a molecular weight of 781.73, but this dye is a mixture of colored and uncolored compounds, and the impurities contribute to the staining properties.

Absorption characteristics

Dissolve 50 mg chlorazol black E in 250 ml 50% alcohol. Dilute 15 ml of this solution to 200 ml with 50% alcohol. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 597–602 nm; P–15/P+15 ratio, between 0.97 and 1.00.

Assay

No method for quantitative analysis has been devised for chlorazol black E.

Biological test

Chlorazol black E is tested on paraffin sections of animal tissue fixed in Zenker's fluid, and on plant tissue fixed in Flemming's or Bouin's fluid. The sections are stained 5–10 min in a 1% solution of the dye in 70% ethyl alcohol. Excess dye is drained off and the sections are dehydrated, cleared, and mounted in a resinous medium.

The tissue elements should stain varying shades of green, gray and black, and sharp differentiation should be evident. A generalized green appearance is undesirable.

Congo red (C. I. 22120)

Congo red is the disodium salt of biphenyl-4,4'-disazo-bis-1-naphthylamine-4-sulfonic acid, $C_{32}H_{22}N_6O_6S_2Na_2$.

Absorption characteristics

Dissolve 50 mg Congo red in 250 ml water. Dilute 10 ml of this solution and 2 ml of 1% Na_2CO_3 to 200 ml with water. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 495–500 nm; P–15/P+15 ratio, between 0.99 and 1.04.

Assay

Dissolve 100 mg dye in 170 ml water, add 30 ml 30% sodium tartrate ($Na_2C_4H_4O_6 \cdot 2H_2O$) solution, heat to boiling, and titrate with 0.05 N $TiCl_3$. The end point is sharp, a nearly colorless solution. The following data are used to calculate the percentage of dye.

Molecular weight	696.696
Hydrogen equivalents per mole of dye	8
Milliliters of N/10 $TiCl_3$ per gram of dye	114.827

For certification, samples of this dye must contain $\geq$ 85% dye.

Biological tests

Congo red is tested as a counterstain to alum-hematoxylin, and in one of two methods for amyloid.

For testing as a counterstain to alum-hematoxylin, paraffin sections of animal tissue fixed in Bouin's fluid are dewaxed, hydrated and stained for 5 min in Mayer's hemalum. They then are dipped in tap water once or twice, transferred directly into 0.5% aqueous solution of Congo red for 1 min. The

stained sections then are rinsed in tap water, run up through alcohols, cleared, and mounted.

Congo red should give a bright cytoplasmic stain with some variation from orange to reddish. Erythrocytes, for example, should be light orange and the spindle fibers in mitotic figures should be deep orange to light red.

Congo red is also tested as a stain for amyloid in paraffin sections of formaldehyde fixed human kidney known to contain amyloid deposits using either Highman's or Puchtler's technique.

Highman's amyloid stain. Deparaffinize slides and bring to 50% alcohol. Stain in Congo red solution (Congo red, 0.5 g; 50% alcohol, 100 ml) for 5 min. Differentiate briefly in 0.2% potassium hydroxide in 80% alcohol. Wash in tap water and counterstain with Mayer's alum-hematoxylin (see hematoxylin below). Wash in tap water, dehydrate, clear and mount.

Amyloid stains red and nuclei blue (Highman 1946). Birefringence of the stained amyloid is not a criterion for certification.

Puchtler's amyloid stain (modified by Churukian 2000a). Deparaffinize and hydrate slides. Place in Weigert's iron hematoxylin solution (see hematoxylin below) for 1 min. Wash with water and treat with acid-alcohol (0.5% concentrated hydrochloric acid in 70% alcohol) for 10 sec. Wash well with tap water and rinse in 50% alcohol. Stain in Congo red solution for 20 min (Congo red, 0.1 g; 50% alcohol, 100 ml; sodium chloride, 2.5 g; 1% aqueous sodium hydroxide, 1.0 ml). Rinse rapidly in 95% and 100% alcohol. Clear in xylene and mount with synthetic resin.

Amyloid stains red and nuclei blue. Birefringence of the stained amyloid is not a criterion for certification.

Cresyl violet acetate

Stains with this name are assumed to be cresyl violet acetate, $C_{18}H_{15}N_3O_3$, (F.W. 321), but the name has been applied to other oxazine dyes (see Chapter 19 in Horobin and Kiernan 2002).

Absorption characteristics

Dissolve 50 mg cresyl violet acetate in 125 ml 95% alcohol and dilute to 250 ml with water. Dilute 5 ml of this solution to 200 ml with 50% alcohol. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 595–600 nm; P–15/P+15 ratio, between 0.94 and 1.20.

Assay

Dissolve 100 mg dye in 300 ml 50% alcohol. Add 10 g sodium bitartrate ($NaHC_4H_4O_6 \cdot H_2O$), heat to boiling, and titrate with 0.05 N $TiCl_3$ to a yellow end point. The following data are used to calculate the percentage of dye in the original sample.

Molecular weight	321.342
Hydrogen equivalents per mole of dye	2
Milliliters of N/10 $TiCl_3$ per gram of dye	62.239

For certification, samples should contain $\geq 65\%$ dye content.

Biological test

Cresyl violet acetate is tested as follows. Deparaffinize and hydrate sections of formaldehyde fixed central nervous tissue (spinal cord or brain) and of a specimen containing connective tissue or cartilage. Stain for 10 min in cresyl violet solution (cresyl violet acetate, 0.02 g; water, 100 ml; glacial acetic acid, 0.25 ml). Rinse in three changes of water. Dehydrate in graded alcohols, clear in xylene and mount.

Nissl bodies and nuclei stain blue. Cartilage and mast cell granules stain metachromatically (purple).

Crystal violet (C.I. 42555)

Crystal violet is the chloride of hexamethylparosaniline, $C_{25}H_{30}N_3Cl$.

Absorption characteristics

Dissolve 50 mg crystal violet in 250 ml water. Dilute 2 ml of this solution to 200 ml with water. Read in spectrophotometer in a 1 cm cell. Absorption maximum, 589–594 nm; P–15/P+15 ratio, between 0.98 and 1.20.

Spectrophotometric assay

Percentage of dye = $D\text{-peak} \times 209$. D-peak (color density at peak) is measured using the dye solution described under absorption characteristics above. For certification, samples of this dye should contain $\geq 90\%$ dye.

Biological tests

Crystal violet is tested by four procedures: the Gram stain, the Flemming triple stain with iodine, Newton's crystal violet-iodine technique, and Lieb's amyloid stain.

Gram stain for bacteria. The crystal violet staining solution consists of 2 g crystal violet dissolved in 20 ml 95% alcohol, mixed with 80 ml 1% aqueous ammonium oxalate. Gram's iodine is a solution of

1% iodine and 2% potassium iodide in water. The safranin counterstain contains 0.25 g safranin O in 10 ml 95% alcohol and 100 ml water.

Smears are prepared from a mixture of Gram-negative and Gram-positive bacteria, such as *Escherichia coli* and *Staphylococcus aureus* and fixed by heating the slide over a flame. Flood the horizontal slide with the crystal violet solution for 1 min. Wash in tap water 1–5 sec, then pour on Gram's iodine. After 1 min, wash in tap water, blot dry with filter paper, flood with 95% alcohol for 30 sec and blot dry again. Pour on the safranin counterstain, wait 10 sec, wash in tap water for 1–5 sec, shake the slide and allow it to air dry.

With satisfactory samples of crystal violet and safranin O, the Gram-positive organisms are deep violet and the Gram-negative organisms red.

Flemming triple stain with iodine. This is a method for staining chromosomes in paraffin sections of botanical material (Margolena 1935). Onion root tips or small flower buds are fixed for 12 h in freshly made CRAF fixative (10% aqueous chromium trioxide, 3 ml; 10% acetic acid, 20 ml; formalin [37–40% HCHO], 10 ml; water, 67 ml). Paraffin sections are stained for 1.5–3 h in 1% aqueous safranin O, rinsed in water, treated for 15–25 sec with an alcoholic iodine solution (1% iodine and 1% KI in 80% alcohol), then stained for 5 min in a 1% solution of the crystal violet (or methyl violet) sample. Another rinse in water is followed by another treatment for 30 sec with the alcoholic iodine solution. The slides then are dehydrated in absolute alcohol, differentiated in a 0.25% solution of orange G in clove oil (for about 15 min, checking with a microscope, until mitotic nuclei are red and interphase nuclei are blue-purple), cleared in xylene and mounted in a resinous medium.

A good stain should produce red chromosomes and nucleoli. The nonmitotic chromatin should stain deep purple, and cytoplasm and cell walls should be orange.

Newton's crystal violet-iodine technique (Clark 1981, modified from Newton 1925). Hydrated paraffin sections of onion root tips or small flower buds (fixed as described for Flemming's triple stain) are stained for 10 min in a 1% aqueous solution of either crystal violet or methyl violet, rinsed in water, and treated for 30 sec with an alcoholic iodine solution (1% iodine and 1% KI in 80% alcohol). They are next rinsed in absolute alcohol, differentiated in a saturated solution of orange G in clove oil for 1–2 min or longer until the violet dye is present only in nuclei. The sections then are cleared in xylene and mounted.

With this technique, chromatin and nucleoli should stain deep violet on a light orange background of cytoplasm and cell walls.

Lieb's amyloid stain (Lieb 1947, Churukian 2000a). A saturated stock solution of crystal violet (or methyl violet) is prepared by adding 14 g of dye to 100 ml 95% alcohol. For the working solution, 10 ml of stock is added to 310 ml 95% alcohol followed by 1.0 ml concentrated hydrochloric acid.

For staining, deparaffinize and hydrate sections of a formaldehyde fixed kidney known to contain amyloid. Stain in the working crystal violet solution for 5 min, wash in running tap water for 5 min, rinse in water, and mount in Apathy's aqueous mounting medium.

Note: Apathy's mountant is prepared as follows:

Acacia (gum Arabic)	50 g
Sucrose	50 g
Water	150 ml

Heat to 55–60° C on a heated magnetic stirrer. It usually takes 5–6 h for the acacia to dissolve completely. Then add 10 g sodium chloride and 0.1 g thymol. Store in a refrigerator at 3–6° C.

Amyloid is stained purplish violet and other tissue elements are stained blue.

Darrow red

Darrow red is aminonaphthoacetylaminophenoxazonium chloride, $C_{18}H_{14}N_3O_2Cl$.

Absorption characteristics

Dissolve 50 mg Darrow red in 125 ml 95% alcohol, then dilute to 250 ml with water. Dilute 5 ml of this solution to 200 ml with 50% alcohol. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 503–505 nm; P–15/P+15 ratio, between 1.01 and 1.04.

Assay

Dissolve 100 mg dye in 300 ml 50% alcohol. Add 10 g sodium bitartrate ($NaHC_4H_4O_6 \cdot H_2O$), heat to boiling, and titrate with 0.05 N $TiCl_3$ to a yellow end point. The following data are used to calculate the percentage of dye in the original sample.

Molecular weight	339.791
Hydrogen equivalents per mole of dye	2
Milliliters of N/10 $TiCl_3$ per gram of dye	58.860

For certification, samples should contain $\geq 65\%$ dye.

Biological test

Darrow red is tested for staining neuron cell bodies in paraffin or frozen sections of formalin fixed brain and spinal cord (Powers et al. 1960). Prepare a staining solution by dissolving 50 mg Darrow red in 200 ml 0.2 M acetic acid (pH 2.7), boiling gently for 10 min, then cooling to room temperature and filtering. Stain either frozen sections or dewaxed and hydrated paraffin sections for 20–30 min. Rinse in water, differentiate, and dehydrate through 50, 70, and 95% alcohols. Complete the dehydration in 100% alcohol, clear in xylene, and mount in synthetic resin.

In satisfactory preparations, Nissl bodies and nuclear chromatin are stained red.

Eosin B (C.I. 45400)

Eosin B (eosin bluish) is the sodium salt of dibromodinitrofluorescein, $C_{20}H_6N_2O_9Br_2Na_2$.

Absorption characteristics

Dissolve 50 mg eosin B in 250 ml water. Dilute 5 ml of this solution and 2 ml of 1% Na_2CO_3 solution to 200 ml with water. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 515–520 nm; P–15/P+15 ratio, between 0.96 and 1.22.

Assay

Precipitate the color acid of a weighed sample of the dye to be tested by the method outlined for the analysis of eosin Y. From the weight of the color acid, the dye content of the original sample is calculated by the following formula:

$$\text{Percentage of dye} = \frac{\text{weight of color acid} \times 624.090 \times 100}{580.132 \times \text{weight of original sample}}$$

Note: molecular weight of eosin B = 624.090; molecular weight of the color acid of eosin B = 580.132.)

For certification, samples must contain $\geq 85\%$ dye.

Biological tests

Eosin B is tested as the counterstain for alum-hematoxylin and in Lillie's modified Nocht (azure-eosin) technique.

Counterstain for alum-hematoxylin. Material fixed in either Bouin's or Helly's fluid is embedded in paraffin. Sections are mounted on slides with Mayer's albumen. They are then run down to water, stained 5–10 min in Mayer's hemalum (see under hematoxylin below), dipped once or twice in tap water, then holding them with forceps, dipped

into the solution of eosin B for 5 sec, dehydrated, and mounted. The solution of eosin B is a 0.5% solution of the dye in 20% alcohol. If it overstains, dilute with 2 volumes of water.

A satisfactory sample of eosin B stains cytoplasm, collagen and erythrocytes in shades of pink that differ slightly from those seen when eosin Y is used.

Eosin B is also tested in Lillie's modified Nocht's method (see under azure A below).

Eosin Y (C.I. 45380)

Eosin Y (eosin yellowish) is the sodium salt of tetrabromofluorescein, $C_{20}H_6O_5Br_4Na_2$.

Absorption characteristics

Dissolve 50 mg eosin Y in 250 ml water. Dilute 5 ml of this solution and 2 ml 1% Na_2CO_3 solution to 200 ml with water. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 515–518 nm; P–15/P+15 ratio, between 1.21 and 1.77.

Assay

Dissolve 0.5 g eosin Y in approximately 300 ml water, heat to boiling, and add slowly with constant stirring 3–4 ml of 6 N hydrochloric acid. Cool the solution to room temperature and allow to stand at least 2 h. Filter the precipitated color acid on a weighed sintered glass crucible, thoroughly wash with a 0.2% solution of hydrochloric acid, dry at 110° C, and weigh. Calculate the dye content of the original sample from the weight of the color acid using the following formula:

$$\text{Percentage of dye} = \frac{\text{weight of color acid} \times 691.914 \times 100}{647.948 \times \text{weight of original sample}}$$

Note: molecular weight of eosin Y = 691.914; molecular weight of the color acid of eosin Y = 647.948.

For certification, samples must contain $\geq 88\%$ dye.

Biological tests

Eosin Y is tested for four biological purposes: as a counterstain following a hemalum (alum-hematoxylin) nuclear stain (see under hematoxylin below), as an ingredient of a Wright's stain made up in the laboratory, in a modified Nocht's method (see under azure A above), and in the eosin-methylene blue culture medium for bacteria.

Eosin Y as a counterstain. A 0.5% solution in water or a 0.25% solution in 95% alcohol is used as the counterstain in alum-hematoxylin and eosin (H & E)

procedures with either Mayer's or Harris' hemalum made from an already certified sample of hematoxylin as the nuclear stain.

With a satisfactory eosin Y, red blood cells, eosinophil granules, Paneth cell granules, pancreatic zymogen granules and keratin should be bright orange-red. Other cytoplasm appears in shades of pink, and collagen fibers are lighter pink than smooth and striated muscle fibers (Drury and Wallington 1967, p. 130).

Preparation of Wright's stain. The staining mixture is made from two dyes. For testing, one of these must be from an already certified batch. Add 0.5 g sodium bicarbonate to 100 ml water in a flask, heat gently, and add 0.9 g methylene blue. Put in a boiling water bath for 30 min, shaking occasionally, then cool. Dissolve 0.5 g eosin Y in 500 ml water and filter the methylene blue solution into it. Shake several times during the day and let stand at room temperature overnight. The following day, without shaking the flask until toward the end, filter, then wash with water. Let the washed precipitate dry on its filter paper in a 37° C incubator.

The procedure to be followed is given under Wright's stain below together with a description of the expected appearance in the stained blood film. In judging eosin Y as a component of this stain, special attention is given to whether the red cells show the desired orange-red color. Correct coloration of the granules of the various leukocytes requires satisfactory samples of both dyes used as starting materials for the production of Wright's stain.

Eosin-methylene blue culture medium. This medium is prepared as follows: peptone, 1 g; K₂HPO₄, 0.2 g; agar, 1.5 g; and water, 100 ml. Dissolve by heating and add the following: lactose, 1.0 g; 2% aqueous eosin Y, 2.0 ml; and 0.5% aqueous methylene blue, 1.3 ml. After sterilizing, pour into Petri dishes, inoculate with *Escherichia coli* and *Aerobacter aerogenes*, and allow to incubate 24 h.

With good samples of both dyes there is red growth with a strong metallic sheen for *E. coli* and pink growth without a metallic sheen for *A. aerogenes*. There should be no reddening of the medium except where growth has occurred.

Erythrosin B (C.I. 45430)

Erythrosin B is the sodium salt of tetraiodofluorescein, C₂₀H₆O₅Na₂I₄

Absorption characteristics

Dissolve 50 mg erythrosin B in 250 ml water. Dilute

5 ml of this solution and 2 ml 1% Na₂CO₃ solution to 200 ml with water. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 524–527 nm; P–15/P+15 ratio, between 1.15 and 1.55.

Assay

Precipitate the color acid of a weighed sample of the dye to be tested by the method outlined for the analysis of eosin Y. From the weight of the color acid, the dye content of the original sample is calculated using the following formula:

$$\text{Percentage of dye} = \frac{\text{weight of color acid} \times 879.890 \times 100}{835.924 \times \text{weight of the original sample}}$$

Note: molecular weight of erythrosin = 879.890; molecular weight of the color acid of erythrosin = 835.924.

Samples of this dye should contain ≥ 80% dye.

Biological tests

Erythrosin B is tested in the Jackson stain for plant anatomy, as a counterstain to alum-hematoxylin, and as a substitute for phloxine B in Kreyberg's method for keratin and mucus.

Jackson stain for plant anatomy (Jackson 1926). To employ this procedure as a test for erythrosin, plant material containing both lignified and nonlignified cell walls (wheat stem is convenient) is fixed in CRAF (10% aqueous chromium trioxide, 3 ml; 10% acetic acid, 20 ml; formalin [37–40% HCHO], 10 ml; water, 67 ml), embedded in paraffin, and sectioned. After removing the paraffin, sections are rinsed with absolute alcohol, then 95% alcohol, then stained 15 min in 1% aqueous crystal violet. After quickly rinsing in water and dehydrating, sections are differentiated for 1–5 min in a saturated solution of erythrosin B in clove oil, cleared in 1:1 absolute alcohol:xylene, passed through xylene, and mounted.

By this method the nonlignified tissues should stain pink to red with erythrosin and the lignified cell walls purple with crystal violet.

Counterstain for alum hematoxylin. A 0.5% aqueous solution of erythrosin B is used in place of an eosin Y solution, following Mayer's or Harris' hemalum (see under hematoxylin below).

The shades of red in cytoplasm, collagen and erythrocytes are somewhat different from those obtained with eosin Y, which is usual counterstain in the H & E method.

Kreyberg's method. For a description of this technique, see under phloxine B below.

Ethyl eosin (C.I. 45386)

Ethyl eosin is the potassium or sodium salt of the ethyl ester of tetrabromofluorescein, $C_{22}H_{11}O_5Br_4K$ (or Na).

Absorption characteristics

Dissolve 50 mg ethyl eosin in 250 ml 50% alcohol. Dilute 5 ml of this solution and 2 ml 1% Na_2CO_3 solution to 200 ml with 50% alcohol. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 530–533 nm; P–15/P+15 ratio, between 1.27 and 1.92.

Spectrophotometric assay

Percentage of dye = D-peak $\times$ 130. D-peak (color density at peak) is measured for the dye solution described above under absorption characteristics. For certification, samples must contain $\geq 80\%$ dye.

Biological test

Ethyl eosin is tested in the Stovall and Black method for demonstrating Negri bodies in the central nervous system of rabid animals.

Pieces of hippocampus, cerebral cortex and cerebellum, 3–5 mm in thickness, are placed between squares of ordinary writing paper (cut end next to the paper) and immersed in acetone for 2–6 h. The paper is removed and the fixed tissue is placed in fresh paraffin at 60–62° C for 4 h or overnight. Sections are cut 5 μ m thick, floated onto albumenized glass slides, fixed by gentle heat over a Bunsen burner, and placed in oven for 1 h at 60–62° C. They are washed in two changes of xylene, passed through two changes of absolute and two changes of 95% alcohol, then to water.

The sections are stained for 2 min in 1% ethyl eosin in 95% alcohol adjusted to give a pH meter reading of 4.6 by adding 1 ml of acetate-acetic acid buffer (sodium acetate, 25 g; glacial acetic acid, 5 ml; water, 250 ml). They then are washed in water, stained 1 min in a solution of 0.3 g methylene blue in 30 ml 95% alcohol mixed with 90 ml of water, adjusted to pH 4.6 by adding 1 ml acetate-acetic acid buffer to 60 ml of staining fluid, washed in water, and treated in water acidified with acetic acid (3 drops of glacial acetic acid in 60 ml of water) until the sections become brownish red, rinsed in water, differentiated by passing slowly through 95% alcohol, dehydrated in absolute alcohol, cleared in xylene, and mounted.

By this technique, the Negri bodies are stained terra cotta to cardinal red, nuclei are dark blue, and the cytoplasm pale blue with majority of the tissue (neuropil and myelin) pink.

Ethyl green (C.I. 42590) ("methyl green")

This is the zinc chloride double salt of ethylhexamethylpararosaniline chlorobromide, $C_{27}H_{35}N_3ClBr + ZnCl_2$. As explained in Chapter 14 of Horobin and Kiernan (2002), true methyl green (C.I. 42585), which was invariably contaminated with crystal violet, has not been available commercially for several years. Ethyl green, which is not usually contaminated with crystal violet, is a superior product, but it is nevertheless frequently sold under the name of methyl green.

Absorption characteristics

Dissolve 50 mg ethyl green ("methyl green") in 250 ml water. Dilute 10 ml of this solution to 200 ml with water. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 630–635 nm; P–15/P+15 ratio, between 0.86 and 1.11. Dilute the solution just prior to reading because its properties change with time. Take the peak reading first, then the ratio points as rapidly as possible.

Assay

Dissolve 200 mg dye in 100 ml 95% alcohol. Add 100 ml water and 10 g sodium bitartrate ($NaHC_4H_4O_6 \cdot H_2O$), heat to boiling and titrate with 0.05 N $TiCl_3$ to a greenish yellow end point. The following data are used to calculate the percentage of dye in the original sample.

Molecular weight	653.237
Hydrogen equivalents per mole of dye	2
Milliliters of N/10 $TiCl_3$ per gram of dye	30.615

For certification, samples must contain $\geq 80\%$ dye content.

Biological tests

Ethyl green is tested as a counterstain in Altmann's aniline-acid fuchsin technique for mitochondria, and in the ethyl green-pyronine method for differential coloration of nucleic acids.

Altmann's aniline-acid fuchsin technique for mitochondria. Altmann's staining solution is prepared as follows. Add a few drops of aniline to 25 ml water. Shake vigorously and filter. Dissolve 1 g acid fuchsin in 10 ml of the aniline-water. Wait overnight, and filter the solution into a dropping bottle. The ethyl green counterstain is a 1% aqueous solution of ethyl ("methyl") green.

Paraffin sections are prepared from formaldehyde fixed kidney. The paraffin is removed and the sections are hydrated in the usual manner. The sections on a horizontal staining rack are covered

with a few drops of Altmann's staining solution and carefully heated over a flame until steaming. They are then allowed to cool while the staining continues for 4–6 min. The slides are drained and covered with water. After 3 min, they are drained again, then covered for 6–50 sec with the 1% aqueous ethyl green counterstain. The time in ethyl green must be adjusted to give adequate counterstaining of nuclei without excessive extraction of acid fuchsin. The counterstained slides are drained, plunged into 95% alcohol for 1 sec, rinsed in absolute alcohol, cleared in xylene, and mounted in a resinous medium.

With satisfactory samples of acid fuchsin and ethyl green, mitochondria and zymogen granules are red and nuclei are green.

Ethyl green is also tested in the ethyl green-pyronine stain for DNA and RNA (see under pyronine Y below).

Fast green FCF (C.I. 42053)

Fast green FCF is the disodium salt of an acid triphenylmethane dye, $C_{37}H_{34}O_{10}N_2S_3Na_2$.

Absorption characteristics

Dissolve 200 mg fast green FCF in 250 ml 50% alcohol. Dilute 3 ml of this solution to 200 ml with 50% alcohol. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 622–626 nm; P–15/P+15 ratio, between 0.98 and 1.20.

Assay

Dissolve 100 mg dye in 200 ml water, add 3 g sodium bitartrate ($NaHC_4H_4O_6 \cdot H_2O$), heat on a hot plate to 95° C, and titrate with 0.05 N $TiCl_3$. The end point varies in color from yellow to reddish brown. The following data are used to calculate the percentage of dye.

Molecular weight	808.875
Hydrogen equivalents per mole of dye	2
Milliliters of N/10 $TiCl_3$ per gram of dye	24.726

Samples of this dye should contain $\geq$ 85% dye.

Biological tests

Fast green FCF is tested as a cytological counterstain and as a component of a one-step trichrome method.

Safranin-fast green FCF stain for plant cells (based on Haynes 1928). Onion root tips or small flower buds are fixed for 12 h in freshly made CRAF fixative (10% aqueous chromium trioxide, 3 ml; 10% acetic acid, 20 ml; formalin [37–40% HCHO], 10 ml; water,

67 ml). Hydrated paraffin sections are stained in 1% aqueous safranin for 30–50 min, rinsed in water and differentiated with 0.2% fast green FCF in 95% alcohol until the chromatin and nucleoli remain red. The slides are then passed through absolute alcohol to xylene and mounted in a synthetic resinous medium.

Chromosomes and other chromatin bodies, as well as nucleoli and lignified cell walls (if present), should appear bright red. Spindles, cellulose cell walls, and cytoplasm are green.

Gomori's one-step trichrome stain (Gomori 1950). This technique is applied to paraffin sections of animal material that contains connective tissue. Lung, kidney, intestine and muscle, fixed in formaldehyde or Bouin's fluid are suitable objects. Deparaffinize slides and place them for 10 min in Bouin's solution preheated to 58° C (saturated aqueous picric acid, 75 ml; concentrated formalin, 25 ml; glacial acetic acid, 5 ml). Wash in running tap water until yellow color disappears. Place in Weigert's iron hematoxylin (see under hematoxylin below) for 10 min to stain nuclei. Wash in water and place for 15 min in Gomori's (1950) trichrome stain, which contains:

0.6 g chromotrope 2R (C.I. 16570)
0.3 g fast green FCF or light green SF or aniline blue
0.8 g phosphotungstic acid
100 ml water
1 ml glacial acetic acid

Rinse 2 min in 0.5% aqueous acetic acid, dehydrate, clear and mount.

Muscle and erythrocytes should stain red, collagen green or blue according to the dye used, and nuclei black.

Fluorescein Isothiocyanate (FITC)

FITC is tested for its capacity to form a fluorescent conjugate with protein in immunofluorescence technology. The test employed is that described by Cherry et al. (1969), which involves a determination of the fraction of the dye sample that conjugates with a standard protein (bovine serum albumin; BSA) under standard conditions. This fraction, expressed as percent, is termed the labeling efficiency of the sample. An arbitrary value of 70% has been adopted as the minimum acceptable for certification. This test has also been adopted by the National Committee for Clinical and Laboratory Standards (NCCLS, 771 E. Lancaster Ave., Villanova, PA 19085, and was published in detail in June, 1975, as Approved Standard: ASM-1, "Stan-

dard Test for Labeling Efficiency of Fluorescein Isothiocyanate (FITC)." The technical instructions for determining labeling efficiency are given in that document (NCCLS 1975).

The labeling efficiency is printed on the BSC certification label, together with the date of testing. The latter is important because FITC is light- and moisture-sensitive and can be expected to deteriorate with prolonged storage, especially if it is not in a refrigerated desiccator (see Chapter 16 in Horobin and Kiernan 2002).

Giemsa stain

Like Wright's stain, Giemsa stain is formed by mixing eosin with polychromed methylene blue.

Absorption characteristics

Stains containing eosin Y and methylene blue and its derivatives have absorption maxima at 518 nm (eosin) and often slightly higher and broader at about 660–665 nm, indicating the predominant presence of methylene blue and azure B, respectively.

Chromatography

TLC is performed to examine the composition of the mixture (Fig. 6), and HPLC is also employed to examine the composition of the dye sample. The results of these tests do not currently constitute criteria for certification by the BSC.

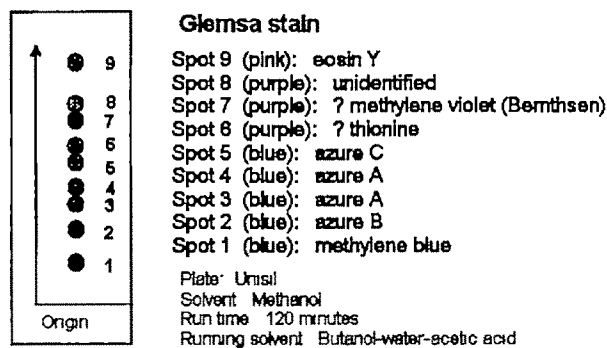


Fig. 6. TLC of a sample of Giemsa's stain.

Biological tests

Giemsa stain for blood films. Giemsa is tested on dried thin films of blood that have been treated 2–7 min with methyl alcohol. To prepare the Giemsa stock solution, 0.5 g of the powdered dye mixture is dissolved in 33 ml glycerol by heating at 55–60° C for 1–2 h. Thirty-three milliliters of methyl alcohol are added, and the solution allowed to stand 24 h. The blood films, dried after the methanol fixation, are immersed in a staining fluid containing 30 drops

(0.67 ml) of the stock solution above in 30 ml water, and also in 30 ml of pH 6.8 phosphate buffer. Different lots of the stain vary in their speed of action. Samples are therefore tested with three or four slides stained for periods varying from 15 to 40 min. A sample is not rejected for certification if good results are obtained on any one of the slides.

Results should be closely similar to those obtained with a good Wright's stain; the chief difference is that with Giemsa the nuclei of leukocytes are reddish purple instead of a dark violet.

Wolbach's Giemsa method (Luna 1968). To be accepted for certification, the Giemsa stain must also prove satisfactory for tissue staining with Wolbach's Giemsa method as follows: Deparaffinize and hydrate sections of formaldehyde fixed bone marrow. Place in acid alcohol (70% alcohol, 995 ml; concentrated hydrochloric acid, 5 ml) for 10 min. Wash well in tap water. Place in working Giemsa solution (Giemsa stock solution, 1 ml; methyl alcohol, 1.5 ml; water, 40 ml) overnight. Immerse in 1% acetic acid for 5 sec. Rinse in water and dehydrate quickly in three changes of 95% alcohol and four changes of absolute alcohol. Clear in xylene and mount with synthetic resin.

With a satisfactory sample of Giemsa's stain Wolbach's method produces blue nuclei, purple mast cells, pink eosinophils, pink to red collagen and muscle (if present), and blue bacteria (if present).

Hematoxylin (C. I. 75290)

Absorption characteristics

Spectrophotometric analysis is not currently used for the evaluation of hematoxylin in the BSC laboratory.

Assay

Spectrophotometric assays for hematoxylin and its oxidation product hematein (Marshall and Horobin 1974b) are under development in the BSC laboratory, but are not yet used for certification of samples of hematoxylin.

Biological tests

Hematoxylin is tested on paraffin sections of animal tissue fixed in any general fixative. Weigert's iron hematoxylin, Mayer's acid hemalum, Harris' hemalum and Heidenhain's technique are used.

Weigert's iron hematoxylin with Van Gieson counter-stain. This method is used for testing hematoxylin and acid fuchsine. One or the other of these dyes

must be from an already certified sample. The Weigert's iron hematoxylin working solution is prepared by mixing a 1% solution of hematoxylin in 95% alcohol with an equal volume of 0.012% ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) in water containing 1% by volume concentrated hydrochloric acid. Van Gieson's picro-fuchsin is made by mixing 10 ml of 1% aqueous acid fuchsin with 100 ml saturated aqueous picric acid.

Sections are passed through xylene and alcohol to water, then stained for 10 min in the working Weigert's iron hematoxylin solution, rinsed in water, and counterstained for 3–5 min in Van Gieson's picro-fuchsin for 3 min. Sections then are passed through three changes of 95% alcohol and three changes of absolute alcohol, cleared, and coverslipped using a synthetic resinous mounting medium.

A satisfactory hematoxylin should stain nuclei a crisp deep blue or black.

Mayer's hemalum with eosin Y counterstain. To prepare the staining solution, dissolve 1 g hematoxylin in 140 ml water, then add 10 g aluminum ammonium sulfate [ammonium alum, $\text{NH}_4\text{Al}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$] and 100 mg sodium iodate (NaIO_3). When these have dissolved, add 60 ml glycerol and 4 ml glacial acetic acid. Glycerol replaces the chloral hydrate of the original Mayer's hemalum formulation. Run sections down to water in the usual manner and stain for 5 min in the hematoxylin solution above, rinse and blue in tap water, counterstain in 0.25% eosin Y in 95% alcohol, rinse in tap water, dehydrate, clear, and mount in a resinous medium.

A satisfactory hematoxylin should stain the nuclei blue. This coloration is shifted toward purple by the eosin counterstain. Other elements of the tissue appear in shades of pink.

Harris' hemalum with eosin Y counterstain. This staining solution is prepared as follows. Dissolve 0.25 g hematoxylin in 2.5 ml 95% alcohol. Dissolve 5 g aluminum ammonium sulfate [ammonium alum, $\text{NH}_4\text{Al}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$] in 50 ml water with heating. Mix the two solutions, bring to a boil and add 0.3 g sodium iodate (NaIO_3). As soon as the solution assumes a dark purple color, remove the heat and cool by plunging flask into cold water. Before use, add 1 ml glacial acetic acid to each 25 ml of staining solution. In this variant of Harris' hemalum, 0.3 g NaIO_3 replaces 0.125 g mercuric oxide (HgO) in the original formulation.

Remove paraffin in the usual manner and stain the slides for 1 min in the working Harris' hematoxylin solution. Rinse in tap water until

sections are blue, then counterstain in 0.25% eosin Y in 95% alcohol. Rinse in water, dehydrate in two changes each of 95% and 100% alcohol, clear, and mount in a synthetic resinous medium.

With a satisfactory sample of hematoxylin, nuclei are colored dark purple.

Heidenhain's technique. The paraffin is removed and the slides are hydrated in the usual manner. The sections are immersed in 1.5–4% aqueous ferric ammonium sulfate (iron alum; $\text{NH}_4\text{Fe}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$) for 30 min to 3 h, rinsed in tap water, and stained for 1–3 h in a 0.5% aqueous solution of hematoxylin that has been allowed to mature for 2 days. The slides are next rinsed in tap water, and differentiated in the iron alum solution above under visual control by microscopic examination until there is obvious black nuclear staining and a clear background. The stained sections then are washed in running water for 5–10 min, counterstained with 1% aqueous orange G (from a certified sample of that dye) for 2–5 min, dehydrated, cleared, and mounted in Canada balsam or a synthetic resin.

With careful control of the differentiation, this technique can demonstrate many intracellular structures. For testing purposes, however, differentiation is continued until the nuclei of cells are the only objects stained. With a satisfactory sample of hematoxylin the nuclei should appear black.

Indigocarmin (C.I. 73015)

Indigocarmin is the disodium salt of indigotin-5,5'-disulfonic acid, $\text{C}_{16}\text{H}_8\text{N}_2\text{O}_8\text{S}_2\text{Na}_2$.

Absorption characteristics

Dissolve 50 mg indigocarmin in 250 ml water. Dilute 10 ml of this solution to 200 ml with water. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 608–612 nm; P–15/P+15 ratio, between 0.98 and 1.07.

Assay

Dissolve 200 mg dye in 200 ml water, add 15 g sodium bitartrate ($\text{NaHC}_4\text{H}_4\text{O}_6 \cdot \text{H}_2\text{O}$), heat to boiling, and titrate with 0.05 N TiCl_3 . The color change at the end point is from blue to yellow. The following data are used to calculate the percentage of dye in the original sample.

Molecular weight	466.370
Hydrogen equivalents per mole of dye	2
Milliliters of N/10 TiCl_3 per gram of dye	42.884

For certification, samples should contain $\geq 80\%$ dye.

Biological test

Indigocarmine is tested using Cajal's picro-indigocarmine method on Bouin fixed intestine and kidney, and on formaldehyde fixed lung. Deparaffinize and hydrate sections, and stain in Orth's lithium carmine solution (see under carmine above) for 5 min. Transfer to acid alcohol (1% concentrated hydrochloric acid in 70% alcohol) to differentiate, leaving red in the nuclei. Wash in tap water, and stain with picro-indigocarmine solution (indigocarmine, 0.25 g; saturated aqueous picric acid, 100 ml) for 10 min. Dehydrate and differentiate with alcohol. (The sections become green in absolute alcohol.) Clear in xylene and mount.

Nuclei are stained red, connective tissue blue-green, and muscle greenish yellow.

Janus green B (C.I. 11050)

Janus green B is *N,N*-dimethylaminophenyl-azo-*N,N*-diethylphenosafranine hydrochloride, $C_{30}H_{31}N_6Cl$.

Absorption characteristics

Dissolve 50 mg Janus green B in 125 ml 95% alcohol, then dilute to 250 ml with water. Dilute 10 ml of this solution to 200 ml (10 mg/l) with 50% alcohol. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 655–665 nm; P–15/P+15 ratio, between 0.98 and 1.03.

Assay

Dissolve 100 mg of dye in 50 ml 95% alcohol, add 150 ml water and 10 g sodium bitartrate ($NaH-C_4H_4O_6 \cdot H_2O$), heat to boiling, and titrate with 0.05 N $TiCl_3$.

The dye solution undergoes several color changes upon reduction, but the final end point, usually yellow or orange-yellow, is easy to recognize. The following data are used to calculate the percentage of dye in the original sample.

Molecular weight 511.083

Hydrogen equivalents per mole of dye 6

Milliliters of N/10 $TiCl_3$ per gram of dye 117.397

Samples of this dye should contain $\geq 50\%$ dye.

Biological test

Janus green B, together with neutral red, is tested for supravital staining of blood. Stock solutions of the two dyes are prepared by adding 75 mg neutral red to 25 ml absolute alcohol and 50 mg Janus green

B to 25 ml absolute alcohol. Both solutions are stable. Just before using, a diluted mixed stain is prepared by adding 35 to 40 drops of neutral red stock and 5 drops of Janus green B stock to 5 ml absolute alcohol.

Store chemically cleaned slides in 95% alcohol; wipe dry, avoiding lint. Warm the slide and flood rapidly with the diluted mixed stain. Drain and air dry. Place a drop of fresh human blood on a coverslip and invert this onto the top of the slide. Seal the edges of coverslip with white petrolatum (petroleum jelly, Vaseline). Let the preparation stand for at least 5 min before placing on the stage of a microscope. Examine at room temperature; however, for observing cellular motility, the optimum temperature is 37° C.

With good samples of both dyes, basophil granules are stained deep brick red, eosinophil granules are yellow or light orange, neutrophil granules are pale pink, and vacuoles in monocytes are salmon-pink. Mitochondria are green. All the cells that stain with neutral red take up the dye quickly except monocytes, which require about 10 min. The mitochondria become apparent after about 15 min as small green dots or rods. The cells should remain in good condition for at least an hour.

Jenner's Stain

Jenner's stain is a methylene blue eosinate and is obtained as the precipitate formed by mixing solutions of methylene blue and eosin Y. It thus resembles Wright's stain, but differs from the latter in not using polychromed methylene blue; for this reason is generally considered inferior to Wright's stain.

Absorption characteristics

Stains containing eosin Y and methylene blue and its derivatives have absorption maxima at 518 nm (eosin) and often slightly higher and broader at about 650–656 nm, indicating the presence of methylene blue and azure B, respectively.

Chromatography

HPLC is employed to examine the composition of the dye sample. TLC also is used to show the composition of the mixture (Fig. 7).

Biological tests

A solution of 0.25 g Jenner's stain powder is made up in 100 ml of methyl alcohol. This is used to stain blood smears as described under Wright's stain.

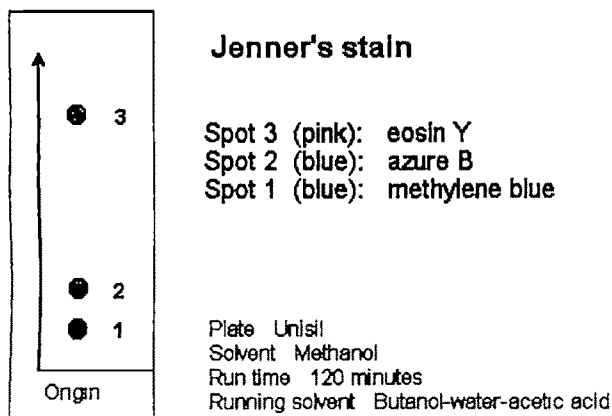


Fig. 7. TLC of a sample of Jenner's stain.

Nuclei are a lighter, more greenish blue than nuclei stained with Wright's stain, which are purple-violet.

Jenner's stain is also tested in Nocht's method by using 5 ml of the methanolic solution in place of separate eosin and azure solutions (see under azure A above).

Light green SF (C.I. 42095)

Light green SF yellowish is the disodium salt of an acid triphenylmethane dye, $C_{37}H_{34}N_2O_9S_3Na_2$.

Absorption characteristics

Dissolve 50 mg light green SF in 250 ml water. Dilute 5 ml of this solution to 200 ml with water (5 mg/l). Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 629–634 nm; P–15/P+15 ratio, between 0.96 and 1.16.

Assay

Dissolve 200 mg of dye in 200 ml water, add 10 g sodium bitartrate ($NaHC_4H_4O_6 \cdot H_2O$), heat on a hot plate to 65° C, and titrate with 0.05 N $TiCl_3$. The end point is a sharp change from light green to yellow. The following data are used to calculate the percentage of dye in the original sample.

Molecular weight	792.875
Hydrogen equivalents per mole of dye	2
Milliliters of N/10 $TiCl_3$ per gram of dye	25.225

Samples of this dye should contain $\geq 65\%$ dye.

Biological test

Light green SF is tested as a cytological counterstain to safranin and as a component of Gomori's one-step trichrome method. See under fast green FCF above for descriptions of these methods.

Malachite green (C.I. 42000)

Malachite green is the chloride or oxalate of a basic triphenylmethane dye, $C_{23}H_{25}N_2Cl$ or $2C_{23}H_{25}N_2 + 3C_2H_2O_4$.

Absorption characteristics

Dissolve 50 mg malachite green in 250 ml water. Dilute 3 ml of this solution to 200 ml with water. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 616–620 nm; P–15/P+15 ratio, between 1.00 and 1.15.

Test for anion

Samples of malachite green are usually labeled as chloride or oxalate, but the identity of the anion can be checked with a simple chemical test. Dissolve approximately 0.1 g of the dye in 50 ml cold water and precipitate the dye base by adding a slight excess of dilute NaOH. Filter off the base and divide the filtrate into two parts. Heat one part to boiling and add saturated aqueous $Ca(OH)_2$ solution (lime-water). A fine white crystalline precipitate indicates that the original dye was the oxalate. Make the other portion of the filtrate slightly acid with dilute HNO_3 and add a dilute aqueous solution of $AgNO_3$. A copious white precipitate forms if the original dye contained chloride ions.

Assay

Dissolve 200 mg of dye in 120 ml water, add 50 ml alcohol, 10 ml glacial acetic acid and 30 ml 30% sodium tartrate ($Na_2C_4H_4O_6 \cdot 2H_2O$) solution, heat to boiling, and titrate slowly with 0.05 N $TiCl_3$ to a light straw color. The following data are used to calculate the percentage of dye.

Malachite green chloride

Molecular weight, $C_{23}H_{25}N_2Cl$	364.926
Hydrogen equivalents per mole of dye	2
Milliliters of N/10 $TiCl_3$ per gram of dye	54.806

Malachite green oxalate

Molecular weight, $2C_{23}H_{25}N_2 + 3C_2H_2O_4$	929.052
Hydrogen equivalents per mole of dye	4
Milliliters of N/10 $TiCl_3$ per gram of dye	43.054

For certification, samples should contain $\geq 75\%$ dye if the chloride, or $\geq 90\%$ dye if the oxalate.

Biological tests

Malachite green is tested as a counterstain for botanical material (preferably pathological, such as wheat with stem rust), after fixation in a suitable fixative (e.g., CRAF, Flemming's, Carnoy's, or Farmer's fluid) and embedment in paraffin. The paraffin is removed in the usual manner.

Stain hydrated sections for 20 min in 1% aqueous safranin O and wash in water. Apply a few drops of 0.5% malachite green in 95% alcohol for 20 sec, pour off the stain, rinse quickly with 1 drop of 100% alcohol, clear in xylene, and mount.

When used on plant pathological material, the nuclei, xylem, cutinized walls and the nuclei of infecting fungus should appear red. The cytoplasm and cellulose walls of the host should appear green.

Malachite green is also tested as a Schaeffer and Fulton (1933) spore stain for bacteria. Smears are prepared of bacteria (*Bacillus cereus*, incubated for 24 h, then left at room temperature for one day), fixed over a flame, flooded with 5% aqueous malachite green for 30–60 sec, and heated to steaming three or four times. Smears are washed in water for 30 sec and 0.5% aqueous safranin then is added for 30 sec. The stained smears are finally washed in water, blotted and left to dry.

The spores should appear green and the other cells red.

Martius yellow (C.I. 10315)

Martius yellow is the monosodium or calcium salt of 2,4-dinitro-1-naphthol, $C_{10}H_5N_2O_5Na$ or $(C_{10}H_5N_2O_5)_2Ca$.

Absorption characteristics

Dissolve 50 mg martius yellow in 250 ml water. Dilute 10 ml of this solution to 200 ml with water. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 431–440 nm; P–15/P+15 ratio, between 1.00 and 1.12.

A marked distinction is observed in the solubilities of the two salts in water. The sodium salt is soluble to the extent of 4.5 parts in 100 parts of water at 26° C, whereas only 0.05 part of the calcium salt is soluble in 100 parts of water at the same temperature.

Assay

Dissolve 100 mg dye in 200 ml water, add 10 g sodium bitartrate ($NaHC_4H_4O_6 \cdot H_2O$), heat to boiling, and titrate with 0.05 N $TiCl_3$. A change from orange to a straw yellow indicates the end point. The following data are used to calculate the percentage of dye in the sample.

Molecular weight, $C_{10}H_5N_2O_5Na$	256.157
Hydrogen equivalents per mole of dye	12
Milliliters of N/10 $TiCl_3$ per gram of dye	468.456
Molecular weight, $(C_{10}H_5N_2O_5)_2Ca$	506.402
Hydrogen equivalents per mole of dye	24
Milliliters of N/10 $TiCl_3$ per gram of dye	473.930

An acceptable minimum dye content has not yet been established for martius yellow.

Biological tests

This dye is tested in a mixture with lacmoid (resorcin blue) for staining pollen tubes in dissected or crushed unfixed styles of flowers (Nebel 1931). Martius yellow provides a background counterstain to the blue color imparted to callose, a structural polysaccharide of pollen tubes (Ruzin 1999).

Slender styles and ovaries are crushed while still moist between two slides; larger ones are treated similarly after sectioning longitudinally by hand. The material is either stained on the slide or immersed in pieces in the stain for 2–5 min. The staining solution consists of 5 mg lacmoid or iris blue (C.I. 51400) and 5 mg martius yellow in 10–15 ml water with a few drops of 1% aqueous ammonia added to bring the reaction to about pH 8 as indicated by the solution assuming an olive color. The material is mounted in the stain or in water, and examined under a powerful light.

A good sample is one in which the pollen tubes are blue on a light yellowish green background.

Martius yellow is also tested as a counterstain for alum hematoxylin. Staining for 1–2 min in a 0.5% aqueous solution imparts a yellow color to tissue components other than nuclei.

Methyl orange (C.I. 13025)

Methyl orange is the sodium salt of *p*-dimethylaminoazobenzene sulfonic acid, $C_{14}H_{14}N_3O_3SNa$.

Absorption characteristics

Dissolve 50 mg methyl orange in 250 ml water. Dilute 5 ml of this solution and 1 ml of 1 N HCl to 200 ml with water. Read in a spectrophotometer in a 1 cm cell. Adsorption maximum, 505–508 nm; P–15/P+15 ratio, between 0.95 and 0.97.

Assay

Dissolve 100 mg of dye in 200 ml water, add 15 g sodium bitartrate ($NaHC_4H_4O_6 \cdot H_2O$), heat to boiling, and titrate with 0.05 N $TiCl_3$. The end point is sharp, giving a colorless solution. The following data are used to calculate the percentage of dye in the original sample.

Molecular weight	327.347
Hydrogen equivalents per mole of dye	4
Milliliters of N/10 $TiCl_3$ per gram of dye	122.194

Samples of this dye should contain $\geq 85\%$ dye.

Biological tests

Methyl orange is tested using the Wilson-Ezrin method for pituitary acidophils (see under orange G below), Kreyberg's method for keratin (see under phloxine B below), as a counterstain, applied for 1-2 min following Heidenhain's or Weigert's hematoxylin on paraffin sections of animal tissue fixed in Zenker's fluid, and as a substitute for orange G in Flemming's triple stain (see under crystal violet above).

A satisfactory sample of methyl orange shows a good contrast to the nuclear stain.

Methyl violet 2B (C.I. 42535)

Methyl violet is a mixture of the more highly methylated fuchsines, principally pentamethylparosaniline chloride. The formula $C_{24}H_{28}N_3Cl$ is employed for purposes of estimation.

Absorption characteristics

Dissolve 50 mg methyl violet in 250 ml 50% alcohol. Dilute 2 ml of this solution to 200 ml with 50% alcohol. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 583-587 nm; P-15/P+15 ratio, between 1.06 and 1.19.

Spectrophotometric assay

Percentage of dye = $D_{\text{peak}} \times 207$. D_{peak} (color density at peak) is measured on the dye solution described above under absorption characteristics. Samples should contain $\geq 75\%$ dye.

Biological tests

Methyl violet is tested by the same procedures as crystal violet (C.I. 42555; see above), but omitting the Gram stain. Results should be the same except that the color has a less red hue with methyl violet than with crystal violet.

Methylene blue (C.I. 52015)

Methylene blue is bis(dimethylamino)phenothiazine hydrochloride (tetramethylthionine), $C_{16}H_{18}N_3SCl$, usually in crystals with $3H_2O$. This dye is sometimes marketed as the zinc chloride double salt, but it is not recommended in that form for use as a biological stain.

Absorption characteristics

Dissolve 50 mg methylene blue in 250 ml water. Dilute 3 ml of this solution to 200 ml with water. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 663-667 nm; P-15/P+15 ratio, between 1.21 and 1.70.

Chromatography

TLC is performed to examine the composition of the dye (Fig. 8). HPLC, also performed to test sample purity, should show that $\geq 70\%$ of the dye is methylene blue.

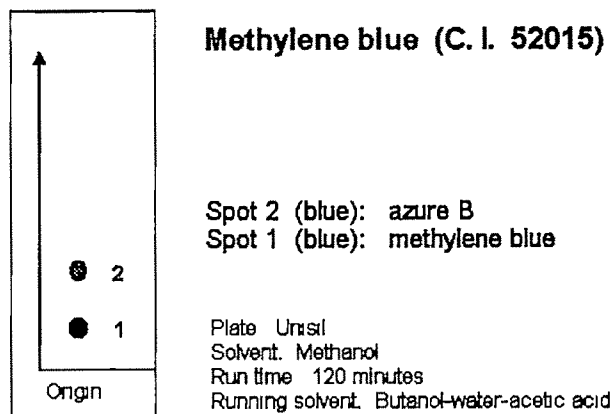


Fig. 8. TLC of a sample of methylene blue.

Assay

Dissolve 100 mg of dye in 200 ml water, add 10 g sodium bitartrate ($NaHC_4H_4O_6 \cdot H_2O$), heat to boiling, and titrate with 0.05 N $TiCl_3$ to a pale yellow end point. The following data are used to calculate the percentage of dye in the original sample.

Molecular weight	319.867
Hydrogen equivalents per mole of dye	2
Milliliters of N/10 $TiCl_3$ per gram of dye	62.526

For certification, samples must contain $\geq 82\%$ anhydrous dye.

Biological tests

Methylene blue is tested for histological and bacteriological staining, and as a constituent of Wright's blood stain.

Histological stain. As a histological stain, this dye is tested in Mallory's phloxine-methylene blue on paraffin sections of tissue fixed in Zenker's fluid, with certified phloxine as a counterstain. For technical instructions, see under phloxine B below.

A good sample of methylene blue should show strong blue nuclear staining without removing the pink to red phloxine from the cytoplasm.

Staining bacteria in milk. A 0.01 ml quantity of milk is placed on a microscope slide, smeared over an area of 1 cm^2 with a stiff needle, dried with gentle heat on a level surface, dipped in xylene for a few minutes to remove fat, and immersed in 90-95% ethyl alcohol for a few minutes to fix the smear to

the slide. It is then dipped 2–4 times in methylene blue solution (methylene blue, 0.3 g; 95% ethyl alcohol, 30 ml; after dissolving, mix with 100 ml water), and washed briefly in tap water until the intense blue color becomes faint. The slide is then air dried and examined.

A good sample of methylene blue should not remove the milk smear from the slide, and should show deeply stained bacteria against a light blue background.

Wright's stain. Instructions for making Wright's stain in the laboratory are given under eosin Y above, and the method of use is described under Wright's stain below.

Methylene violet Bernthsen (C.I. 52041)

This oxidation product of methylene blue must not be confused with methylene violet 3RAX (C.I. 50206) or methylene violet RR (C.I. 50205), which are unrelated dyes with different properties and uses.

Absorption characteristics

Dissolve 25 mg methylene violet Bernthsen in 125 ml 95% alcohol, add 10 ml 0.1 N HCl, and dilute to 250 ml with water. Dilute 10 ml of this solution with 20 ml of 0.1 N HCl to 200 ml with 50% alcohol. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 578–582 nm; P–15/P+15 ratio, between 0.99 and 1.06.

Spectrophotometric assay

Percentage of dye = D-peak $\times$ 181. D-peak (color density at peak) is measured using the dye solution described under absorption characteristics above. For certification, samples should contain $\geq$ 65% dye.

Chromatography

HPLC is employed to examine the composition of the dye sample, but the results are not presently used by the BSC for certification.

Biological test

This stain is tested as a constituent of MacNeal's tetrachrome stain. For instructions for preparing MacNeal's tetrachrome from its component dyes, see under MacNeal's tetrachrome stain below.

A blood smear stained with this mixture containing a satisfactory methylene violet Bernthsen shows a pure blue in the cytoplasm of the lymphocytes.

Neutral red (C.I. 50040)

Neutral red is aminodimethylaminomethylphenazine hydrochloride, C₁₅H₁₇N₄Cl.

Absorption characteristics

Dissolve 50 mg neutral red in 250 ml 50% alcohol containing 1.25 ml glacial acetic acid. Dilute 5 ml of this solution to 200 ml with 50% alcohol containing 1 ml of glacial acetic acid. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 540–544 nm; P–15/P+15 ratio, between 1.00 and 1.12.

Spectrophotometric assay

Percentage of dye = D-peak $\times$ 132. D-peak (color density at peak) is measured for the dye solution described under absorption characteristics above. For certification, samples of this dye must contain $\geq$ 50% dye.

Biological test

Neutral red is tested for supravital staining of living blood cells using the same procedure as used for Janus green B above.

Nigrosine, water soluble (C.I. 50420)

Nigrosine, water soluble, is the sodium salt of the product resulting from sulfonation of spirit soluble nigrosine, a mixture of dyes obtained by the interaction of aniline, aniline hydrochloride, and nitrobenzene.

Absorption characteristics

Dissolve 50 mg nigrosine, water soluble in 250 ml 50% alcohol. Dilute 25 ml of this solution to 200 ml with 50% alcohol. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 574–580 nm; P–15/P+15 ratio, between 0.93 and 1.00.

Assay

This dye is a variable mixture of complex compounds of uncertain constitution. Dye content is not determined at this time.

Biological test

Nigrosine, water soluble is tested in Dorner's spore stain, using a 2–4-day-old culture of a rapid spore-former such as *Bacillus cereus*.

A heavy suspension of the organism is prepared in 2–3 drops of water in a small test tube, and an equal quantity of freshly filtered Ziehl's carbol-fuchsin is added. This mixture is allowed to stand in a boiling water bath 10 min or longer. One

loopful of the stained preparation is mixed in a coverslip or slide with a loopful of 5–10% aqueous nigrosine, prepared by placing 1 g nigrosine and 10 ml water in a test tube in boiling water bath for 30 min. (This solution must be filtered before use, and may be kept indefinitely if preserved with a few drops of formalin.) It is smeared as thin as possible and allowed to dry fairly rapidly.

A good sample of nigrosin, water soluble stains spores red, the vegetative cells are unstained, and the background is dark gray.

Nile blue A (Nile blue sulfate) (C.I. 51180)

Nile blue A is aminodiethylaminonaphthophenoxazonium sulfate, $(C_{20}H_{20}N_3O)_2SO_4$. For use in lipid histochemistry, Nile blue A must contain an adequate proportion of its oxidation product, Nile red (see Chapter 19 in Horobin and Kiernan 2002).

Absorption characteristics

Dissolve 50 mg Nile blue A in 125 ml water, then dilute to 250 ml with 95% alcohol. Dilute 3 ml of this solution to 200 ml with 50% alcohol. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 635–640 nm; P–15/P+15 ratio, between 1.02 and 1.16.

Assay

Dissolve 100 mg of dye in 100 ml 95% alcohol. Add 75 ml water and 25 ml 30% sodium tartrate ($Na_2C_4H_4O_6 \cdot 2H_2O$) solution, heat to boiling, and titrate with 0.05 N $TiCl_3$. It is necessary to keep the solution boiling as the end point is approached. The final color change is from reddish brown to yellow. The following data are used to calculate the percentage of dye in the sample.

Molecular weight	732.874
Hydrogen equivalents per mole of dye	4
Milliliters of N/10 $TiCl_3$ per gram of dye	54.584

For certification, samples of this dye should contain $\geq 75\%$ dye.

Biological tests

Nile blue A is tested for staining and discriminating between melanins and lipofuscins in paraffin sections of animal tissue, and in a histochemical method for lipids in frozen sections.

Nile blue method for melanin and lipofuscin (Lillie and Fullmer 1976). Paraffin sections are prepared of formaldehyde fixed fatty liver. Paraffin is removed in the usual manner, and the sections are stained

20 min in a solution consisting of 0.05% Nile blue A in 1% H_2SO_4 (H_2SO_4 98.5%, 1 ml; water, 99 ml), washed 10 min in running water and mounted in glycerol-gelatin.

A satisfactory sample of the dye stains lipofuscins dark blue or green-blue, melanin dark green, cytoplasm and muscle pale green, erythrocytes greenish yellow to greenish blue, and myelin green to deep blue. Transfer some slides directly from the stain into acetone. This decolorizes lipofuscins, but leaves melanin green.

Lillie's sulfuric Nile blue technique for fatty acids. (Lillie and Fullmer 1976). Frozen sections of fatty liver and avocado are stained for 20 min in the same Nile blue A solution as that used for staining melanin and lipofuscin. Sections are washed for 10 min in running water and mounted in glycerol-gelatin.

Fatty acids are dark blue; neutral fats are pink to red.

Oil red O (C.I. 26125)

Oil red O is *p*-xylylazo-*p*-xylylazo-2-naphthol, $C_{26}H_{24}N_4O$ (M.W. 408.51).

Absorption characteristics

Dissolve 50 mg of dye in 250 ml benzene. Dilute 2 ml of this solution with 50 ml benzene. Read in a spectrophotometer in a 1 cm glass cell. Absorption maximum, 518–528 nm; P–15/P+15 ratio, between 0.96 and 1.01.

Chromatography

TLC and HPLC are employed to examine the composition of the dye sample (Fig. 9).

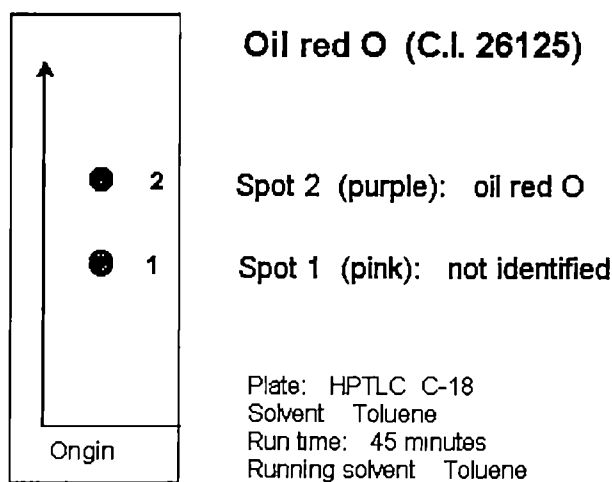


Fig. 9. TLC of a satisfactory sample of oil red O.

Spectrophotometric assay

Percentage of dye = D-peak $\times$ 192. D-peak (color density at peak) is measured for the dye solution described under absorption characteristics above. For certification, samples of this dye should contain $\geq 70\%$ dye.

Biological test

Oil red O is tested by the method of Lillie and Ashburn (1943), modified by Churukian (1999). Prepare a saturated (approximately 1%) stock solution of oil red O in 99% isopropanol. Dilute 60 ml of stock solution with 40 ml 1% dextrin. Any type of dextrin is suitable. Let stand overnight, then filter. The filtrate, which is the working solution, can be used for several weeks. The sections are stained 10 min or longer, rinsed in 60% isopropanol, then in water. Nuclei are counterstained for 5 min in approximately 0.1% acid alum hematoxylin (e.g., Mayer's undiluted or Lillie's diluted 1 to 4 in 2% acetic acid or Ehrlich's diluted 1 to 5 in 2% acetic acid), then placed in 0.3% sodium borate or in tap water until blue. Sections are mounted in a suitable aqueous medium such as glycerin jelly, Apathy's syrup, Zwerner's glychrogel or Kaiser's mounting medium.

A good sample should give the fat globules an orange-red color; nuclei are blue.

Orange II (C.I. 15510)

Orange II is the sodium salt of 2-hydroxy-1-naphthylazobenzene sulfonic acid, $C_{16}H_{11}N_2O_4SNa$.

Absorption characteristics

Dissolve 50 mg orange II in 250 ml water. Dilute 10 ml of this solution to 200 ml with water. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 482–487 nm; P–15/P+15 ratio, between 0.93 and 0.98.

Assay

Dissolve 100 mg of dye in 200 ml water, add 10 g sodium bitartrate ($NaHC_4H_4O_6 \cdot H_2O$), heat to boiling and titrate with 0.05 N $TiCl_3$. Reduce to a nearly colorless solution. The following data are used to calculate the percentage of dye in the original sample.

Molecular weight	350.337
Hydrogen equivalents per mole of dye	4
Milliliters of N/10 $TiCl_3$ per gram of dye	114.179

Samples of this dye should contain $\geq 85\%$ dye.

Biological test

Orange II is tested using the same procedures as those listed for methyl orange above, except that it is not tested in Kreyberg's method.

Orange G (C.I. 16230)

Orange G is the disodium salt of benzenediazo-2-naphthol-6,8-disulfonic acid, $C_{16}H_{10}N_2O_7S_2Na_2$.

Absorption characteristics

Dissolve 50 mg orange G in 250 ml water. Dilute 15 ml of this solution of 200 ml with water. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 476–481 nm; P–15/P+15 ratio, between 0.88 and 0.95.

Assay

Dissolve 100 mg of dye in 200 ml water, add 10 g sodium bitartrate ($NaHC_4H_4O_6 \cdot H_2O$), heat to boiling, and titrate with 0.05 N $TiCl_3$ to a sharp change from brownish yellow to a pale yellowish green. The following data are used to calculate the percentage of dye.

Molecular weight	452.386
Hydrogen equivalents per mole of dye	4
Milliliters of N/10 $TiCl_3$ per gram of dye	88.420

For certification, samples must contain $\geq 80\%$ dye.

Biological tests

Orange G is tested in Mallory's connective tissue stain (see under acid fuchsin above), the Wilson-Ezrin and Kreyberg (see under phloxine B below) methods for pituitary acidophils and keratin, as a counterstain in histology, and for cytology.

Wilson-Ezrin pituitary Stain. Deparaffinize sections and hydrate to water. Oxidize in 0.5% aqueous periodic acid for 10 min. Rinse in water. Place in Schiff's reagent (see basic fuchsin above) for 15 min. Wash in running tap water for 10 min, and stain with Weigert's iron hematoxylin (see hematoxylin above). Treat with 0.5% acid-alcohol (0.5 ml concentrated hydrochloric acid in 100 ml 70% alcohol) for 10 sec and wash in running tap water. Place in 1% aqueous orange G solution for 1.5 min. Treat with 5% aqueous phosphotungstic acid for 30 sec. Wash in running tap water, rinse in distilled water, dehydrate, clear and mount.

Beta granules should stain red, gamma granules purple, acidophils yellow, and nuclei black (Wilson and Ezrin 1954, Churukian 1997).

As a counterstain, a 1% aqueous or 0.2% alcoholic solution of orange G is applied for 15 sec

or more after Heidenhain's iron hematoxylin (see under hematoxylin above).

Structures not stained black by the iron hematoxylin are colored yellow.

For cytological work, orange G is tested in Flemming's triple stain on paraffin sections of onion root tips. The method is described under crystal violet (see crystal violet above).

An acceptable stain gives clear yellow contrast to the darkly purple nuclei and chromosomes.

Orcein

The chemical composition of orcein is discussed in Chapter 19 of Horobin and Kiernan (2002).

Absorption characteristics

Dissolve 50 mg orcein in 250 ml 0.01 N NaOH. Dilute 10 ml of this solution to 200 ml with 0.01 N NaOH. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 575–582 nm; P–15/P+15 ratio, between 0.98 and 1.18.

Assay

Dye content is not determined by the BSC at this time.

Biological tests

Orcein is tested as a stain for elastin and for hepatitis B antigen.

Orcein method for elastic fibers. Orcein is tested as an elastic tissue stain on Zenker, Bouin, or formalin fixed tissue. The paraffin is removed in the usual manner. An iodine-thiosulfate sequence is used to remove mercury deposits from sections of Zenker fixed specimens.

The sections are stained for 30 min in orcein (0.4 g orcein in 100 ml 70% alcohol with 1 ml concentrated HCl), then rinsed briefly in 70% alcohol. The sections then are counterstained for 2 min with Van Gieson's stain (0.025% acid fuchsin in saturated aqueous picric acid), dehydrated with 95% and absolute alcohol, cleared in xylene and mounted.

A satisfactory sample of the dye should stain even the finest elastic fibers either a dark purple or reddish purple. The counterstain produces red collagen and yellow cytoplasm. The orcein elastin color may be deepened to black or reddish black by washing, just after staining, in millimolar FeCl₃ or CuSO₄ in 70% alcohol for a minute or so. Nuclear staining also is intensified, but the background is paler.

Orcein method for hepatitis B surface antigen (Deodhar 1975). Deparaffinize and hydrate sections of formaldehyde fixed liver (biopsy or postmortem material) from a known case of hepatitis B. Place in acidified potassium permanganate (KMnO₄, 0.15 g; water, 100 ml; concentrated sulfuric acid, 0.1 ml) for 5 min. Wash in water and place in 2% aqueous oxalic acid for 2 min. Wash in running tap water for 3 min, rinse in water, and stain for 4–8 h in orcein solution (orcein, 1 g; 70% alcohol, 100 ml; concentrated hydrochloric acid, 1 ml; let stand for 1 week before using). Rinse in water, dehydrate, clear and mount in synthetic resin.

Hepatitis B surface antigen, present diffusely in hepatocyte cytoplasm, stains brown.

Pararosaniline (C.I. 42500)

Pararosaniline is triaminotriphenylmethane. Its chloride has the formula C₂₀H₁₈N₃Cl and its acetate is C₁₉H₁₈N₃·C₂H₃O₂. Pararosaniline is available as a pure dye and it is also a component of basic fuchsin (see Chapter 14 in Horobin and Kiernan 2002).

Absorption characteristics

Dissolve 50 mg pararosaniline in 125 ml 95% ethyl alcohol, shake for 20 min, then dilute to 250 ml with water. Dilute 3 ml of this solution to 200 ml with 50% ethyl alcohol. Read in a spectrophotometer in a 1.0 cm cell. Absorption maximum, 544–547; P–15/P+15 ratio, between 1.15 and 1.36.

Chromatography

HPLC is performed to verify the purity of the dye sample. A single peak is seen if pararosaniline is the only dye of the basic fuchsin family present in the sample. If more than one peak is present, the dye is tested as basic fuchsin rather than pararosaniline.

Assay

First determine whether the sample is a chloride or an acetate using the qualitative test described under basic fuchsin above. Dissolve 100 mg of the dye in 175 ml water, add 25 ml 30% sodium tartrate (Na₂C₄H₄O₆·2H₂O), heat to boiling, and titrate with 0.05 N TiCl₃ to a yellow end point. The following data are used to calculate the percentage of dye in the sample.

	Chloride	Acetate
Molecular weight	323.834	347.423
Hydrogen equivalents per mole of dye	2	2

Milliliters of N/10 TiCl_3 per
 gram of dye 61.760 57.566
 For certification, samples should contain $\geq 88\%$
 dye.

Biological tests

Pararosaniline is tested in the same procedures as basic fuchsin (see under basic fuchsin above). A certified sample of pararosaniline, therefore, is also a certified basic fuchsin.

In addition, pararosaniline is tested in Gomori's aldehyde fuchsin method for pancreatic B cells. This technique also stains the hepatitis B surface antigen. Pararosaniline is the only component of basic fuchsin suitable for this technique (see Chapter 14 in Horobin and Kiernan 2002).

Aldehyde fuchsin method (Shikata et al. 1974). Gomori's aldehyde fuchsin solution is made by mixing the following ingredients. (The dye must be pararosaniline; other components of basic fuchsin are unsuitable.)

Pararosaniline	0.5 g
70% Ethyl alcohol	96.0 ml
Paraldehyde	3.0 ml
Concentrated hydrochloric acid	1.0 ml

Allow the solution to stand ("ripen") for 5 days before using.

The stain is used on mounted paraffin sections of formaldehyde fixed mammalian pancreas and human liver from a known case of hepatitis B. Deparaffinize the slides and process to 70% alcohol. Place in ripened aldehyde fuchsin for 15 min (for hepatitis B surface antigen or for 1 h for pancreatic B cells. Rinse in four changes of water, dehydrate, clear and mount with a synthetic resin.

Pancreatic beta cells and hepatitis B surface antigen are purple. Elastic fibers and laminae also are purple, but arterial elastic fibers of elderly persons may stain weakly or not at all.

Phloxine B (C. I. 45410)

Phloxine B is the sodium salt of tetrabromotetrachlorofluorescein, $\text{C}_{20}\text{H}_2\text{O}_5\text{Cl}_4\text{Br}_4\text{Na}_2$.

Absorption characteristics

Dissolve 50 mg phloxine B in 250 ml 50% alcohol. Dilute 5 ml of this solution with 2 ml 1% Na_2CO_3 solution added to 200 ml with 50% alcohol. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 546–550 nm; P–15/P+15 ratio, between 1.38 and 2.24.

Assay

Precipitate the color acid of a weighed sample of the dye to be tested using the method outlined for the analysis of eosin Y. From the weight of the color acid, the dye content of the original sample is calculated using the following formula:

$$\text{Percentage of dye} = \frac{\text{weight of color acid} \times 829.710 \times 100}{\text{weight of the original sample} \times 785.744}$$

Note: molecular weight of phloxine B = 829.710; molecular weight of the color acid of phloxine B = 785.744.)

Samples of this dye should contain $\geq 80\%$ dye.

Biological tests

Phloxine B is tested in Mallory's phloxine-methylene blue stain and Kreyberg's method for keratin and mucus.

Mallory's phloxine-methylene blue stain. This is carried out with paraffin sections of intestine that have been fixed in Zenker's fluid. The paraffin is removed in the usual manner and the sections are stained 20 min or longer in 2.5% aqueous phloxine B, washed in water, stained for 30 min in borax-methylene blue (Stock solution contains methylene blue, 1 g; borax, 1 g; water, 100 ml. The stock solution is diluted 1:9 with water). The stained slides are washed in water, differentiated and dehydrated in 95% alcohol containing a few drops of a 10% alcoholic solution of rosin (colophony). The slides are kept in constant motion so that the decolorization is uniform. Staining is monitored under the microscope. When the pink color has returned to the section and the nuclei are still deep blue, dehydration is quickly terminated with absolute alcohol, and the sections are cleared in xylene and mounted.

The cytoplasm should be stained pink in contrast to the blue of the nuclei.

Kreyberg's (1961) method for keratin and mucus. Four dyes are used in this staining technique, which is used in the BSC laboratory for testing alcian blue and three red xanthene dyes: erythrosin B, phloxine B and rose Bengal. One of the dyes used in the procedure, metanil yellow, is not assayed or certified by the BSC. Of the former three dyes, two should already be certified and one is the dye being evaluated. The red xanthene dye used in this method may be erythrosin B, phloxine B or rose Bengal. The original technique has been modified by Churukian and Schenk (1984) and Churukian (2000b).

Deparaffinize and hydrate sections of small intestine, trachea and larynx. Place in 3% acetic

acid for 3 min. Stain in 40 ml alcian blue solution (1 g alcian blue and 1 ml acetic acid in 100 ml water) for 3 min in a microwave oven (60 W), and leave in the hot solution with occasional agitation for an additional 5 min. (Without microwave heating, staining in alcian blue requires 30 min at room temperature.) Rinse in water. Place in ammonia-alcohol solution (10 ml ammonium hydroxide [28%] added to 90 ml 95% alcohol) for 10 min. Wash well with tap water and place in Weigert's iron hematoxylin solution (see under hematoxylin above), made from a certified hematoxylin, for 45 sec. Wash with water and treat with acid-alcohol (0.5% hydrochloric acid in 70% alcohol) for 10 sec. Rinse well in water and stain in either phloxine B solution (1% in water) or erythrosin B solution (1% in water) or rose Bengal solution (1% in water) for 5 min. Rinse quickly in water and dehydrate rapidly in 95% and 100% alcohol. Stain in metanil yellow (0.5% dye in absolute alcohol with 1% acetic acid) for 30 sec. Dehydrate, clear and mount.

Keratin stains red, mucus blue-green, nuclei black, collagen orange, muscle shades of red and orange, and erythrocytes red.

Protargol-S (and similar silver proteinates)

Silver proteinates are reaction products of silver nitrate and protein that have been partly hydrolyzed. The name protargol S is used for products that are intended for staining. They are equivalent to strong silver protein of older pharmacopoeias, and contain about 8% silver. Such a compound is a powder or finely granular material, tan to light brown in color, soluble in water, and precipitated from aqueous solutions by alcohol or acetone. A 1% aqueous solution has a pH of 7.5–8.5. Strong silver protein gives a heavy precipitate with NaCl, indicating free silver ions. Mild silver protein has a very low concentration of unbound silver ions, although its total content of silver is higher (Budavari 1996).

Assay

No satisfactory chemical tests are available because those ordinarily used (total silver content, detectable free silver ions etc.) do not correlate with staining performance. Performance of a given sample under actual staining conditions, therefore, is the only dependable criterion.

Biological test

Silver proteinates are tested using a modification of Bodian's protargol method for selective staining of nerve fibers in mammalian brain and spinal cord

(Bodian 1936). The modification is the use of a different developing solution. Specimens may be fixed in Bouin's fluid or in Bodian's (1937) alcoholic formalin-acetic acid (formalin, 5 ml; glacial acetic acid, 5 ml; 80% alcohol, 90 ml).

Paraffin sections are hydrated to distilled water in the usual manner and stained for 12–48 h at 37° C in a 1% aqueous solution of protargol-S previously prepared by dusting the required amount of material on the surface of distilled water and allowing it to dissolve without shaking from the surface downward. Just before placing slides in a Coplin jar for staining, about 5 g of bright clean granular copper is placed in the bottom of the jar. Duplicate slides are also stained in protargol without the addition of copper. After staining, the slides are rinsed in three changes of distilled water, placed for 10 min in a reducing solution (hydroquinone, 1 g; formalin [37–40% formaldehyde], 5 ml; distilled water, 100 ml), rinsed in three changes of distilled water, and toned for 5–10 min in a 1% solution of gold chloride to which 3 drops of glacial acetic acid per 100 ml have been added. Slides then are rinsed in distilled water, placed for 4–6 min in 2% aqueous oxalic acid solution until sections have a faint purple or blue color, rinsed again in three changes of distilled water, and transferred to a 5% aqueous solutions of sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) for 5 min. The sections are then washed thoroughly in tap water, rinsed in distilled water, dehydrated, cleared, and covered.

Satisfactory staining with protargol-S shows axons and neurofibrils black or purplish-black. The cell nuclei are also black.

Pyronine B (C.I.45010)

Pyronine B is the chloride of a complex of two molecules of tetraethyldiaminoxanthenyl chloride with one ferric ion, $\text{C}_{42}\text{H}_{54}\text{Cl}_8\text{Fe}_2\text{N}_4\text{O}_2$.

Absorption characteristics

Dissolve 50 mg pyronine B in 250 ml 50% alcohol. Dilute 5 ml of this solution to 200 ml with 50% alcohol (5 mg/liter). Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 554–556 nm; P–15/P+15 ratio, between 1.40 and 1.68.

Spectrophotometric assay

Percentage of dye = $(\text{D-peak} \times 100)/1.74$. D-peak (color density at peak) is measured using the dye solution described under Absorption characteristics above. For certification, samples must contain $\geq 40\%$ dye.

Biological test

Pyronine B is tested using the ethyl green-pyronine procedure (see under pyronine Y below).

Pyronine Y (C.I. 45005)

Pyronine Y is tetramethyldiaminoxanthenyl chloride, $C_{17}H_{19}N_2OCl$.

Absorption characteristics

Dissolve 50 mg pyronine Y in 250 ml 50% alcohol. Dilute 5 ml of this solution to 200 ml with 50% alcohol (5 mg/l). Read in a spectrophotometer in a 1 cm cell. Absorption maximum 546–551 nm; P–15/P+15 ratio, between 1.23 and 1.70.

Spectrophotometric assay

Percentage of dye = $(D\text{-peak} \times 100)/1.58$. D-peak (color density at peak) is measured USING the dye solution described under Absorption characteristics above. For certification, samples of this dye must contain $\geq 50\%$ dye.

Biological test

Pyronine Y is tested in the ethyl green-pyronine Y stain applied to paraffin sections of a formaldehyde fixed animal or human tissue that contains cells rich in RNA, such as neurons in brain or plasma cells in reactive lymph nodes or multiple myeloma lesions.

The stain is prepared by dissolving 1 g ethyl green in 100 ml 0.2 M acetate buffer, pH 4.2, then adding 0.1 g pyronine Y. Sections are deparaffinized and hydrated. Slides are stained individually for 5 min, rinsed quickly with water, blotted dry with filter paper, dehydrated rapidly in two changes of acetone, passed through acetone-xylene and two changes of xylene, and coverslipped.

The DNA in nuclei is stained blue-green, and RNA in nucleoli and cytoplasm is stained red.

Resazurin

Resazurin is 7-hydroxy-3H-phenoxazin-3-one-10-oxide, sodium salt, $C_{12}H_6NNaO_4$. It is quite different from resazurin G (C.I. 23580) and resazurin B (C.I. 23590), which are disazo dyes obsolete for textiles and not used as biological stains.

Absorption characteristics

Dissolve 50 mg resazurin in 250 ml water containing 2 ml 1% Na_2CO_3 solution. Dilute 3 ml of this solution to 200 ml with water containing 2 ml of 1% solution. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 600–601 nm; P–15/P+15 ratio, between 1.09 and 1.21.

Spectrophotometric assay

Percentage of dye = $D\text{-peak} \times 162$. D-peak (color density at peak) is measured using the dye solution described under absorption characteristics above.

Biological test

The BSC currently conducts no biological test for resazurin.

Rose Bengal (C.I. 45440)

Rose Bengal is the sodium salt of tetraiodotetrachlorofluorescein, $C_{20}H_2O_5I_4Cl_4Na_2$.

Absorption characteristics

Dissolve 50 mg rose Bengal in 250 ml water. Dilute 5 ml of this solution and 2 ml of 1% Na_2CO_3 solution to 200 ml with water. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 544–550 nm; P–15/P+15 ratio, between 1.21 and 1.95.

Assay

The method for the determination of rose Bengal is identical to that of eosin Y except that for calculating the dye content the following formula is to be used:

$$\text{Percentage of dye} = \frac{\text{weight of color acid} \times 1017.65 \times 100}{973.720 \times \text{weight of the original sample}}$$

Note: molecular weight of rose Bengal = 1017.65; molecular weight of the color acid of rose Bengal = 973.720.

For certification, samples of this stain should contain $\geq 80\%$ dye.

Biological tests

Rose Bengal is tested in Conn's technique for bacteria in soil and as an alternative to phloxine B in Kreyberg's stain for keratin and mucus.

Conn's technique for bacteria in soil. Soil known to have an unusually large number of bacteria should be employed in making this test. A suitable sample must be found by trial and error. A drop of soil suspension, made by mixing soil with 5–10 times its weight of 0.015% gelatin in water, is dried onto a slide. While on a flat surface over boiling water this film is stained for 1 min with 1% rose Bengal in 5% aqueous phenol containing 0.01% $CaCl_2$. The amount of $CaCl_2$ is varied if unsatisfactory results are obtained with 0.01% on the theory that increasing its concentration intensifies the action of the stain. A sample of rose Bengal is judged satisfactory if it imparts deep staining of the bacteria without

sufficient coloring of the soil mineral particles or dead organic matter to obscure the microorganisms.

Kreyberg's method. For a description of this technique, see under phloxine B above.

Safranin O (C.I. 50240)

Safranin O is a mixture of dimethyl and trimethyl phenosafranin chlorides: $C_{20}H_{19}N_4Cl$ and $C_{21}H_{21}N_4Cl$, respectively.

Absorption characteristics

Dissolve 50 mg safranin O in 250 ml 50% alcohol. Dilute 3 ml of this solution to 200 ml with 50% alcohol. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 530–534 nm; P–15/P+15 ratio, between 1.10 and 1.32.

Spectrophotometric assay

Percentage of dye = D-peak $\times$ 230. D-peak (color density at peak) is measured using the dye solution described under absorption characteristics above. For certification, samples must contain $\geq 80\%$ dye.

Biological tests

Safranin is tested for its ability to stain chromosomes in the Flemming triple stain, to stain Gram-negative bacteria in the Gram stain, and in combination with light green SF or fast green FCF. The procedures for the Flemming and Gram stains are described under crystal violet above, and staining with safranin O and light green or fast green FCF is described with fast green FCF above.

Sudan black B (C.I. 26150)

The empirical formula for Sudan black B is $C_{29}H_{24}N_6$. The molecular weight is 456.55, and there are two major isomers (see Chapter 14 in Horobin and Kiernan 2002).

Absorption characteristics

Dissolve 50 mg Sudan black B in 250 ml 95% alcohol. Dilute 10 ml of this solution to 200 ml with 95% alcohol. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 598–604 nm; P–15/P+15 ratio, between 0.98 and 1.02.

Assay

A suitable method for determination of dye content has not been established.

Biological tests

Sudan black B is tested for fatty material in bacteria in Burdon's technique (Burdon et al. 1947). Culture

Bacillus cereus on agar slant tubes at room temperature for 18–24 h. Prepare smears and air dry. A solution of 0.3 g Sudan black B in 100 ml 70% alcohol is prepared. After most of the dye has dissolved, shake thoroughly at intervals during the day and allow to stand overnight. Flood the slide with the stain and allow it to remain undisturbed for 10–20 min. Drain off excess stain, blot dry, clear with xylene, and blot dry again. Counterstain with 0.5% aqueous safranin O for 5–10 sec, wash in tap water, blot, and dry.

A satisfactory sample of Sudan black B should stain intracellular fat dark blue-black.

Sudan black B is also tested as a fat stain in the Lillie-Ashburn supersaturated isopropanol technique (see under oil red O above). Sudan black B is substituted for oil red O in the dye solution.

Sudan III (C. I. 26100)

Sudan III is 1-phenylazo-4-phenylazo-2-naphthol, $C_{22}H_{16}N_4O$.

Absorption characteristics

Dissolve 50 mg Sudan III in 250 ml benzene. Dilute 2 ml of this solution to 50 ml with benzene. Read in a spectrophotometer in a 1 cm glass cell. Absorption maximum, 508–512 nm; P–15/P+15 ratio, between 0.94 and 1.01.

Assay

Owing to its low solubility, Sudan III is sulfonated before titration. Place 100 mg Sudan III in a 100 ml volumetric flask, add 3–4 ml 33% oleum (fuming sulfuric acid: H_2SO_4 with 33% additional SO_3), mix, and heat for about a minute in a boiling water bath. Let stand for about 1 h with occasional shaking. Carefully add dropwise a small amount of water. Allow to cool to room temperature and bring the volume up to the mark. Stopper and stir with a magnetic stirrer for 1 h. Unstopper and place the flask in boiling water bath for 1–2 min. Stir while the solution cools for 2 h. Place the flask in an ice bath, and cautiously dilute with cold water while stirring. Pour the 100 ml of sulfonated solution into the titration flask. Add 100 ml water and 30 g sodium bitartrate ($NaHC_4H_4O_6 \cdot H_2O$), heat to boiling, and titrate with 0.05 N $TiCl_3$ to a yellow end point. The following data are used to calculate the percentage of dye.

Molecular weight	352.402
Hydrogen equivalents per mole of dye	8
Milliliters of N/10 $TiCl_3$ per gram of dye	227.013

For certification, samples of this dye should contain $\geq 80\%$ dye.

Biological test

Sudan III is tested as a fat stain. For the method, see under Sudan IV below. Sudan III, however, is not expected to give a coloration as intense as Sudan IV.

Sudan IV (C. I. 26105)

Sudan IV is *o*-tolylazo-*o*-tolylazo-2-naphthol, $C_{24}H_{20}N_4O$.

Absorption characteristics

Dissolve 50 mg Sudan IV in 250 ml benzene. Dilute 2 ml of this solution to 50 ml with benzene. Read in a spectrophotometer in a 1 cm glass cell. Absorption maximum, 519–523 nm; P–15/P+15 ratio, between 0.97 and 1.01.

Assay

The low solubility of this dye interferes with its assay, therefore it is sulfonated before titration. For a description of the procedure, see under Sudan III above. The percentage of dye in the original sample is calculated from the following data.

Molecular weight 380.456

Hydrogen equivalents per mole of dye 8

Milliliters of N/10 $TiCl_3$ per gram of dye 210.274

For certification, samples must contain $\geq 80\%$ dye.

Biological test

Sudan IV is tested on thin frozen sections using Churukian's variant of the Lillie-Ashburn super-saturated isopropanol method. This method is described under oil red O above. Sudan IV is substituted for oil red O in the dye solution.

Tetrachrome stain (MacNeal)

MacNeal's tetrachrome stain is a mixture containing methylene blue, azure A, methylene violet Bernthsen, and eosin Y. It is sold as a pre-mixed powder. When MacNeal's method is used for testing methylene violet Bernthsen for certification, a tetrachrome powder is made in the laboratory by mixing 1.0 g methylene blue chloride, 0.6 g azure A, 0.2 g methylene violet Bernthsen, and 1.0 g eosin Y. All dyes other than the methylene violet Bernthsen must be from batches of dyes already certified by the BSC.

Absorption characteristics

Stains containing eosin Y, and methylene blue and its derivatives give absorption maxima at 518 nm (eosin) and often slightly higher and broader at

about 650–665 nm, indicating the predominant presence of methylene blue and azure B, respectively. Methylene violet Bernthsen, absorption maximum 580 nm, has little or no effect on the spectrum of this mixture of dyes.

Chromatography

TLC is performed to examine the composition of the mixture (Fig. 10) and HPLC is employed to examine the composition of the dye sample. The results of these tests do not currently constitute criteria for certification by the BSC.

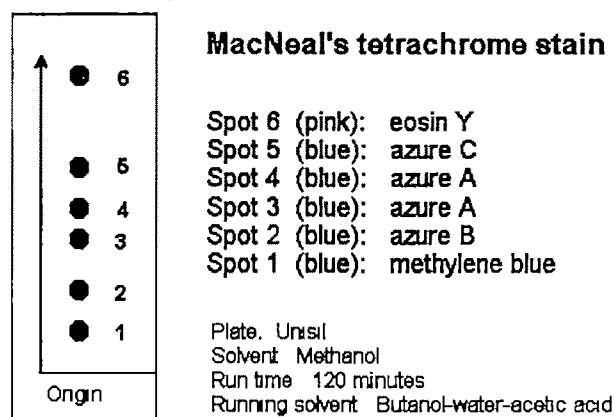


Fig. 10. TLC of a sample of MacNeal's tetrachrome stain.

Biological test

Dissolve 0.30 g MacNeal's tetrachrome stain powder in 100 ml methyl alcohol (neutral, ACS reagent) by heating to 50° C. Shake thoroughly and leave two days at 37° C with occasional shaking. The solution is filtered, then tested by the same procedure as Wright's stain below.

A satisfactory sample of MacNeal's tetrachrome gives results closely similar to those obtained with a good Wright's stain.

Thionine (C.I. 52000)

Thionine is 7-amino-3-iminophenothiazine hydrochloride, $C_{12}H_{10}N_3SCl$.

Absorption characteristics

Add 50 mg thionine to 100 ml water in a 250 ml volumetric flask. Add 2.5 ml glacial acetic acid. Heat for 15 min in a boiling water bath. While still hot, shake for 20 min on a mechanical shaker. Cool to room temperature and dilute to the 250 ml mark with water. Dilute 5 ml of this solution to 200 ml with water. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 598–600 nm; P–15/P+15 ratio, between 1.15 and 1.57.

Spectrophotometric assay

Percentage of dye = D-peak $\times$ 97. D-peak (color density at peak) is measured using the dye solution described under absorption characteristics. Samples should contain $\geq 85\%$ dye.

Biological tests

Thionine is tested as a stain for nervous tissue using the Churukian-Schenk method and for pathological plant material using Stoughton's method.

Churukian-Schenk thionine method

(Churukian and Schenk 1981). This technique is used for evaluating both thionine and toluidine blue. When testing the latter dye, substitute 0.02 g toluidine blue for the 0.02 g thionine in the instructions that follow.

Deparaffinize and hydrate sections of formaldehyde fixed nervous tissue and of an object containing connective tissue or cartilage. Place the slides in 0.5% potassium permanganate solution for 2 min. Rinse in two changes of water and place in 2% potassium metabisulfite for 1 min. Wash in running tap water for 3 min. This pretreatment with KMnO_4 and $\text{Na}_2\text{S}_2\text{O}_5$ minimizes background staining. Stain in thionine solution (thionine, 0.02 g; water, 100 ml; glacial acetic acid, 0.25 ml) for 5 min. Rinse in three changes of water. Dehydrate in graded alcohols, clear in xylene and mount.

Cartilage and mast cell granules stain metachromatically (purple), and Nissl bodies and nuclei stain blue.

Stoughton's method (Stoughton 1930). Pathological plant material (wheat stem rust) is fixed in CRAF (10% aqueous chromium trioxide, 3 ml, 10% acetic acid, 20 ml; formalin [37–40% HCHO], 10 ml; water, 67 ml). Paraffin sections are stained for 1 h in 0.1% thionine in 5% aqueous phenol, dehydrated in successively stronger alcohols, and differentiated in a saturated solution of orange G in absolute alcohol for 1 min. Sections then are washed in absolute alcohol, cleared in xylene, and mounted in a synthetic resin.

Cellulose cell walls should be yellowish or green, and lignified walls blue. The nuclei of the host plant should be blue with purple nucleoli, and deep blue chromosomes. Fungal parasites should be violet to purple, and the nuclei in hyphae or spores deep purple.

Toluidine blue O (C.I. 52040)

Toluidine blue O is 3-amino-7-dimethylamino-2-methylphenazine hydrochloride, $\text{C}_{15}\text{H}_{16}\text{N}_3\text{SCL}$. In

the past it was supplied as a zinc chloride double salt.

Absorption characteristics

Dissolve 50 mg toluidine blue O in 250 ml water. Dilute 5 ml of this solution to 200 ml with water. Read in a spectrophotometer in a 1 cm cell. Absorption maximum, 625–635 nm; P–15/P+15 ratio, between 1.00 and 1.23.

Assay

Dissolve 100 mg of dye in 150 ml water, add 50 ml 95% alcohol and 10 g sodium bitartrate ($\text{NaH}\cdot\text{C}_4\text{H}_4\text{O}_6\cdot\text{H}_2\text{O}$), heat to boiling, and titrate with 0.05 N TiCl_3 to a yellow end point. The following data are used to calculate the percentage of dye in the original sample.

Molecular weight	305.825
Hydrogen equivalents per mole of dye	2
Milliliters of N/10 TiCl_3 per gram of dye	65.394

For certification, samples of this dye must contain $\geq 50\%$ dye.

Biological test

Toluidine blue O is tested as a metachromatic stain using the Churukian-Schenk method as described under thionine above, and in Nocht's method, buffered at pH 4.1, for formalin fixed tissue as described under azure A above.

Wright's stain

Wright's stain is formed by mixing eosin Y with polychromed (oxidized and partly demethylated) methylene blue (see Chapter 21 in Horobin and Kiernan 2002). The following tests are for commercially produced powders composed of the mixed dyes.

Absorption characteristics

Stains containing eosin Y and methylene blue and its derivatives give absorption maxima at 518 nm owing to eosin Y, and often slightly higher and broader absorbance at about 650–665 nm indicating the presence of methylene blue and azure B.

Chromatography

TLC is performed to examine the composition of the mixture (Fig. 11). HPLC also is employed to examine the composition of the dye sample.

Biological tests

The dye mixture (Wright's powder) is dissolved in methanol (0.33 g in 200 ml) and allowed to stand for

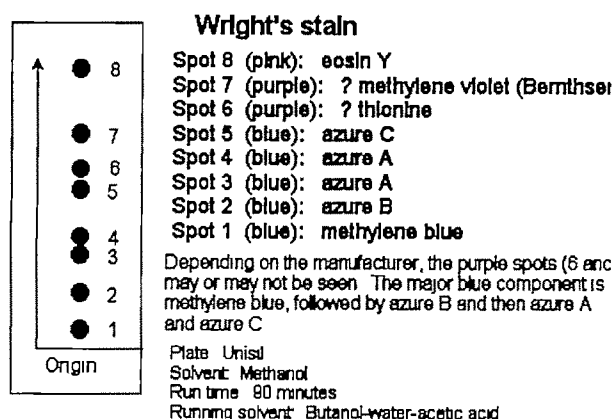


Fig. 11. TLC of a sample of Wright's stain.

5–7 days. The stain is applied with a medicine dropper to a dried human blood film for 1–3 min, after which 1–3 volumes of distilled water or pH 6.4 phosphate buffer are added. After standing twice as long as the undiluted stain, this solution is washed off the slide with distilled water until the thin portions of the stained film are pink. The stained film then is drained and air dried.

A satisfactory sample should show: orange-red erythrocytes; neutrophil leukocytes (polymorphs) with purple nuclei and two types of cytoplasmic granules (dark violet and reddish lilac) and pale pink cytoplasm; eosinophils with violet nuclei, red to orange-red granules, and blue cytoplasm; basophil leukocytes with purple or dark blue nuclei and dark purple (almost black) granules; lymphocytes with dark purple nuclei and sky blue cytoplasm; and platelets with violet to purple granules.

Wright's stain is also tested as a tissue stain in Nocht's method by using 5 ml of 0.16% Wright's stain in methanol in place of the separate eosin and azure solutions (see under azure A above).

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A differential staining method to identify lignified and unlignified tissues

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Abstract

We investigated the use of safranin O and astra blue dissolved in ethyl alcohol as differential stains to distinguish between lignified and unlignified tissues in microtome sections of tension and normal wood of sugar and red maple. Normal wood was used as a control for the histochemical analysis. Lignified and unlignified tissues were found in the same section for both tension and normal wood of each species. These results were confirmed in unstained samples using ultraviolet light. Unlignified libriform fibers were detected using both techniques. Libriform fibers did not fluoresce in UV light, although fluorescence was observed in some of the cell corners. The astra blue in ethyl alcohol and the UV wavelength we used differentiated syringyl from guaiacyl lignins. Ethyl alcohol solutions of these dyes provide an effective and reliable method to distinguish lignified and unlignified tissues.

Key words: astra blue, libriform fibers, normal wood, red maple, safranin O, sugar maple, tension wood

During the past seven decades, great efforts have been made to use dyes to differentiate wood chemical components, especially lignin and cellulose. Lignin constitutes 20–30% of the weight of woody plants and functions as a binding and encrusting material in the cell wall, giving rigidity to the overall plant structure (dos Santos Abreu et al. 1999). Cellulose is composed of glucose monomers linked together into long slender-chain molecules that are about 10 000 sugar units long (Goring and Timell 1962). The cellulose molecules are linked laterally by hydrogen bonds into closely aligned linear bundles exhibiting crystallinity under X-ray and polarized light analysis.

Lignin can be described as a three-dimensional macromolecule originating from phenylpropanoid precursors such as coumaryl, coniferyl, and sinapyl alcohol that are present in vascular plants. In

angiosperms, lignin is found in tracheids, fibers, vessels and parenchyma cell walls (Sarkanen and Ludwig 1971). Despite today's technology, it has not been possible to determine exactly the inter- and intramolecular bonds involving lignin, cellulose and hemicellulose (Carpita and Gibeau 1993). The use of stains, however, makes it possible to prove the presence or absence of lignin and cellulose in wood tissues (Berlyn and Miksche 1976).

There are several dyes that can be used to stain cellulose and lignin. Zinc-chlor-iodide is a common dye that stains cellulose blue, with lignin varying from yellow to orange (Rawlins and Takahashi 1952). The reaction of cellulose with I-KI and H₂SO₄ stains it blue while lignin becomes orange to yellow. One of the most common reagents for lignified tissues is the Mäule reaction that stains lignin red by reacting with phloroglucinol. Safranin O is another dye used to identify lignin, and its reaction product is red.

Histologists know that when some dyes are applied to wood tissues that have been stained previously with another dye, the first stain will be removed and leave the wood tissues colorless if the process is stopped at this stage. If the process is

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allowed to continue, however, the second dye will then stain the tissues by replacing the first dye.

Safranin O is a red basic dye of the azine group that has a positive charge on the dye ion. Basic dyes stain tissue elements that are acidic in reaction, but there are exceptions (Gurr 1960, 1962). Astra blue is a triarylmethane dye (Lillie 1977) that is incorporated into cellulose fibers and stains them blue in the absence of lignin. Safranin O and astra blue in aqueous solution have been used by other authors to visualize the degree of lignification in woody tissues (von Aufsess 1973, Srebotnik and Messner 1994, Sewalt et al. 1997).

In our study, the use of differential staining of sugar and red maple (*Acer saccharum* Marsh. and *Acer rubrum* L.) microtome sections with safranin O and astra blue dissolved in ethyl alcohol was investigated to determine if lignified and unlignified tissues can be detected. Unstained sections also were observed using ultraviolet light to examine lignin fluorescence.

Materials and methods

Three red maple (*Acer rubrum* L.) trees containing tension wood and one tree containing normal wood were selected from ESF's Heiberg Memorial Forest in Tully, NY. One sugar maple (*Acer saccharum* Marsh.) containing tension wood and another sugar maple composed of normal wood also were obtained from Heiberg Forest. Two sugar maple trees containing tension wood were kindly provided by Native American Landscape Company of Syracuse, NY. For each species, the tree containing normal wood was used as a control for histochemical analysis. Wood was stored in the never-dried (green) condition in a freezer.

Small wood blocks for anatomy and histochemical analysis were cut to 10 mm tangential width by 70 mm radial length by 10 mm longitudinal depth. The blocks were placed in vials with distilled water, then put into a glass bell jar to be evacuated for 2 h until they were water-soaked. After soaking, the sugar maple blocks were softened with a 4% ETD (ethylenediamine) solution for 1.5 h at 70° C (Kukachka 1977). Transverse, radial, and tangential sections, 20 µm thick, were cut with a sliding microtome. After dehydration in an ethyl alcohol series, permanent slides of unstained and stained sections were mounted using Histoclad media (No. 3920). Tension and normal wood sections were stained simultaneously in the same watch glass with safranin O (Matheson Coleman & Bell, CI No. 50240) and subsequently with astra blue (6GLL, Aldrich 36, 340-5).

Safranin O was prepared by dissolving 1 g Safranin O in 65 ml 100% ethyl alcohol and 30 ml distilled water. Astra blue was prepared by dissolving 1 g astra blue in 100 ml 100% ethyl alcohol and 5 ml distilled water.

After the sections were stained with safranin O for 2 h, they were rinsed three times in 85% ethyl alcohol for 5–10, which leached some of the dye. Then the samples were stained with the astra blue for 3 min and rinsed quickly three times in 95% and absolute ethyl alcohol. The sections remained in absolute ethyl alcohol for a few minutes until the sections could be placed on slides, then they were cleared with three xylene rinses and mounted in Histoclad medium.

Some sections were stained with safranin O for 2 h, rinsed, and counterstained with fast green FCF (Michrome No. 135, Edward Gurr Ltd.) for 15 sec according to the procedure derived from Gram and Jørgensen (1951). Safranin O and astra blue in aqueous solution also were used to stain some sections following the procedure described by Sewalt et al. (1997). Additional sections were stained with phloroglucinol (493 Eastman Kodak Co.) in HCl using the formula described by Berlyn and Miksche (1976).

A Spot-RT digital camera was used to photograph the sections with a Nikon Optiphot light microscope equipped with DIC and fluorescence optics. Unstained sections also were observed using fluorescence microscopy.

Results and discussion

The double staining of tension wood of sugar and red maple with safranin O and astra blue in aqueous solution produced different shades of red. Previous work (von Aufsess 1973, Srebotnik and Messner 1994) showed that double staining by safranin O and astra blue in aqueous solution stained highly lignified wood fibers pink, whereas less lignified fibers stained red. We observed that the unlignified G-layer in tension wood of red and sugar maple stained blue, the latewood was a lighter red, and the earlywood was a darker red, indicating reduced lignin level (Fig. 1, A–P). A color gradient occurred in the cell corners, which stained a darker red (Fig. 1D,L). Conversely, normal wood stained with safranin O and astra blue in aqueous solution was homogeneously pink, while the middle lamella and cell corners stained darker pink, indicating an increased lignin level (Fig. 1, E–H, M–P). Some areas with highly lignified tissues (fiber tracheids and parenchyma cells) surrounding the

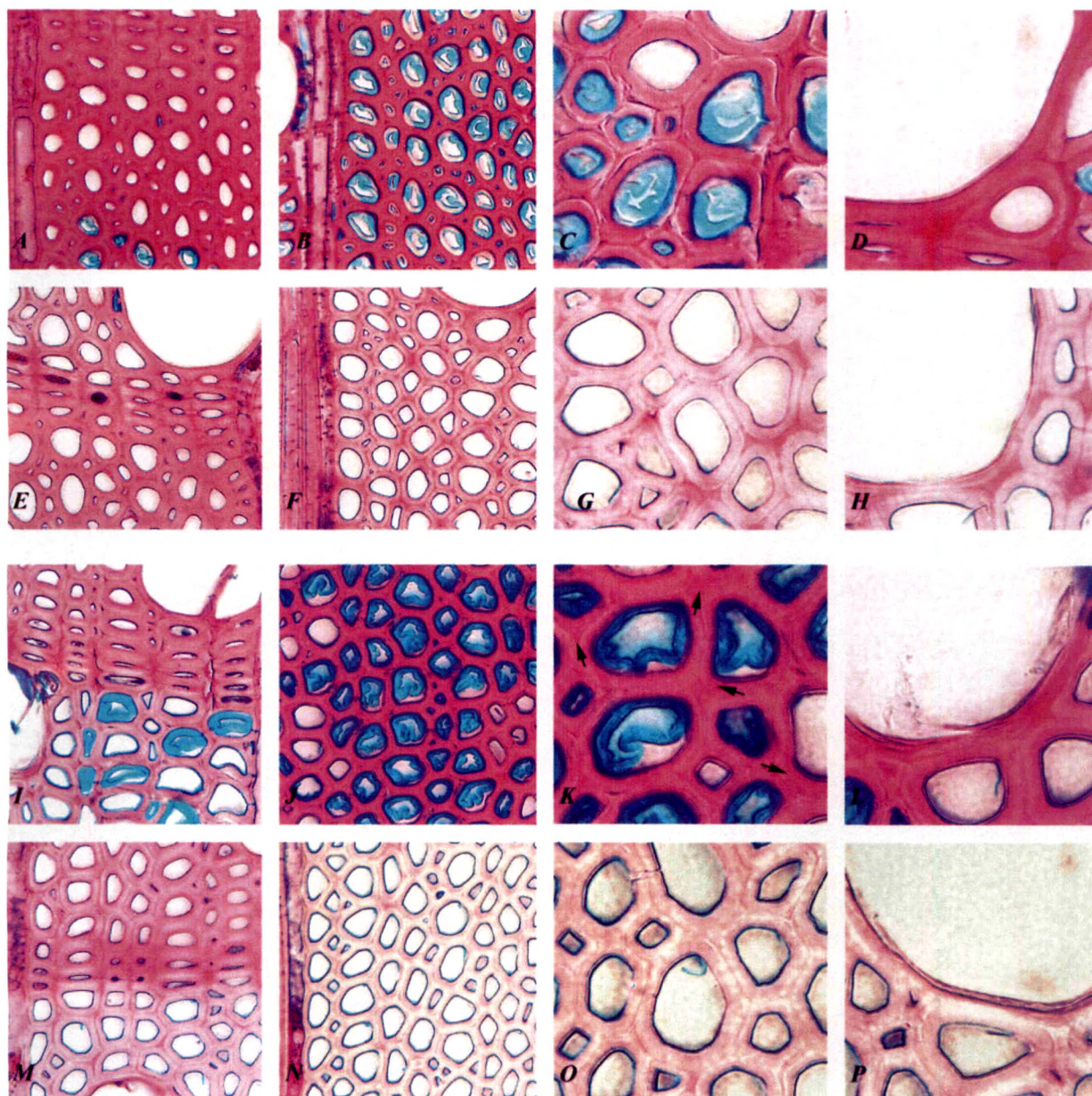


Fig. 1. A–D) Tension wood, and (E–H) normal wood of sugar maple. I–L) Tension wood and (M–P) normal wood of red maple stained with safranin O and astra blue in aqueous solution. Two shades of red in late and early wood can be observed in (A). The tension wood fibers and the cell corners stained darker red (B, D, J, K, L). A gradient in coloration in tension wood fibers is shown in (C). Conversely, normal wood is stained homogeneously pink in both species (E–H, M–P), indicating that the tissue is highly lignified. A, B, E, F, I, J, M, N; $\times 70$; C, D, G, H, K, L, O, P; $\times 165$.

vessel elements also were observed in normal wood of both species (Fig. 1H,P).

The results of the color reaction with phloroglucinol for tension and normal wood of red and sugar maple were similar to those obtained with safranin O and astra blue in aqueous solution, as shown in Figure 1, G,H,O,P. The secondary wall stained pink and the middle lamella stained darker. The ethanol solution of phloroglucinol gave similar results to those observed with aqueous solution of safranin O and astra blue, so the possible influence of the

solvent in the reaction is unknown. The distribution of lignin (or phenolic extracts that could also react with phloroglucinol), however, is supported by the results with phloroglucinol, as demonstrated by the red staining by safranin O.

We noted that unlignified tissues (stained blue) were present in sections stained with safranin O and astra blue in ethyl alcohol solution. These tissues were identified as libriform fibers that occur in undulating bands in both species (Fig. 2A,B, Fig. 3A). Observations of unstained sections with

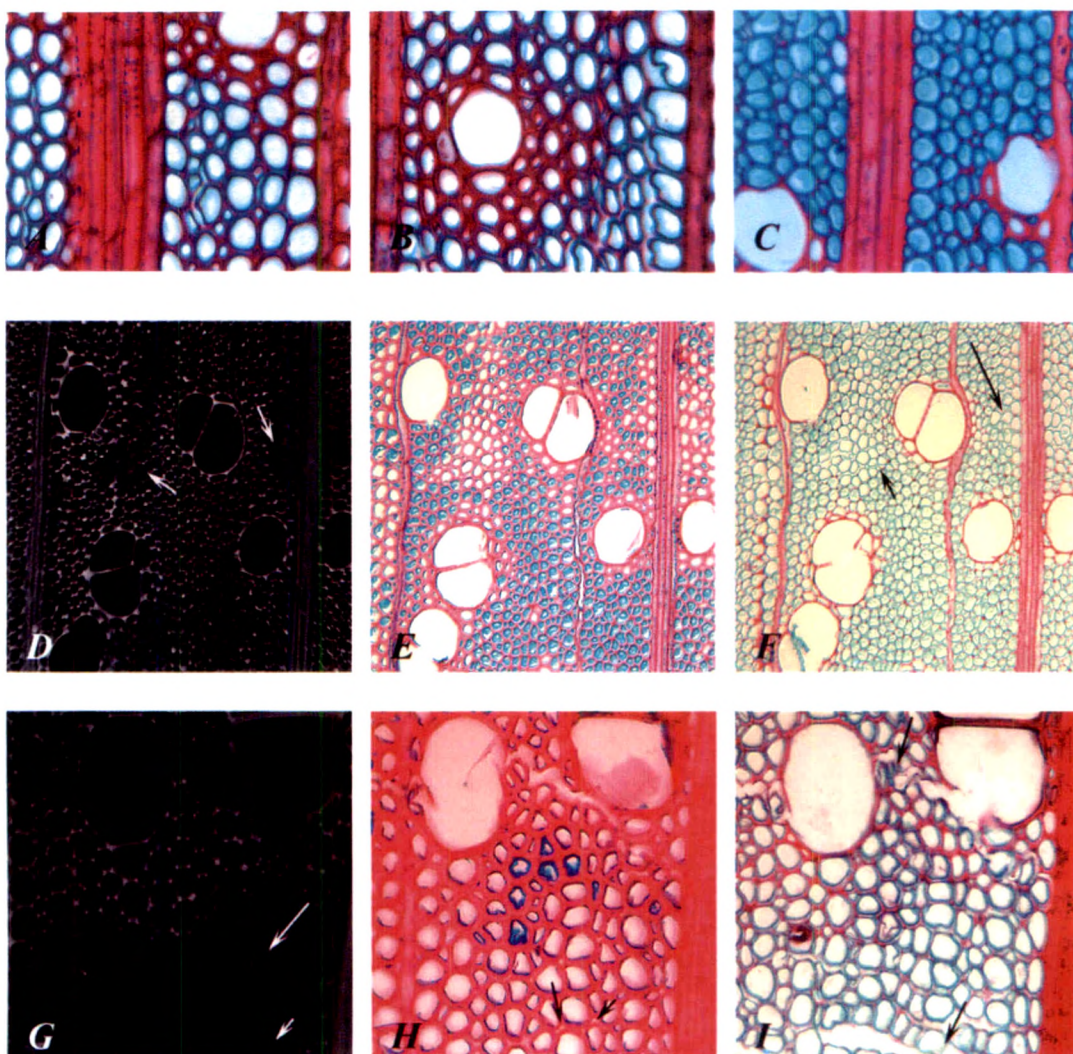


Fig. 2. Tension wood of sugar and red maple. A, B) Differential pattern of unlignified fibers (blue) and lignified fibers (red) in sugar maple. C) Well developed tension wood fibers (G-layer stained blue) that were located just one growth-increment apart from (A) and (B). D–F) Serial sections of tension wood of sugar maple. G–I) Serial sections of tension wood of red maple. (D) and (G) were observed using ultraviolet light (unstained); notice areas with no fluorescence (arrows). (E) and (H) are stained with safranin O and astra blue in aqueous solution; note the tension wood fibers stained blue (G-layer). Note intercellular spaces in (H; arrows). (F) Section stained with safranin O and fast green FCF. Observe that there is not a differential pattern for the libriform fibers (arrows). (I) is stained with safranin O and astra blue in ethyl alcohol solution; note that lignified tissues around the vessels (stained red), and the libriform fibers (stained blue) are separated at the middle lamella (arrow). A–C, $\times 70$; D–F, $\times 25$; G–I, $\times 45$.

ultraviolet illumination revealed that there was no fluorescence in these tissues except in the cell corners (Fig. 2D,G,I, Fig. 3B). It appears that the cell walls contain syringyl, and the cell corners contain syringyl and/or guaiacyl lignin. Other researchers (Srebotnik and Messner 1994, Sewalt et al. 1997) used safranin O and astra blue in aqueous solution to differentiate lignin and cellulose. We used safranin O and astra blue dissolved in ethyl alcohol, resulting in a positive reaction for cellulose (blue color) and lignin (red color). Figure 3A shows an example of this reaction. Areas in blue are nonlignified tissues, which did not fluoresce under

UV light (Fig. 3B). The areas in red are lignified tissues. This differentiating red and blue pattern was not observed when sections were stained with safranin O and astra blue in aqueous solution (Fig. 2E,H) nor when stained with safranin O and fast green FCF (Fig. 2F). These results show that ethyl alcohol solutions of the two dyes are required to differentiate libriform fibers from the surrounding tissue.

Fluorescence is a useful method for detecting lignin and is used for qualitative analysis. Because of the aromatic nature of lignin, it strongly absorbs ultraviolet light and exhibits a characteristic max-

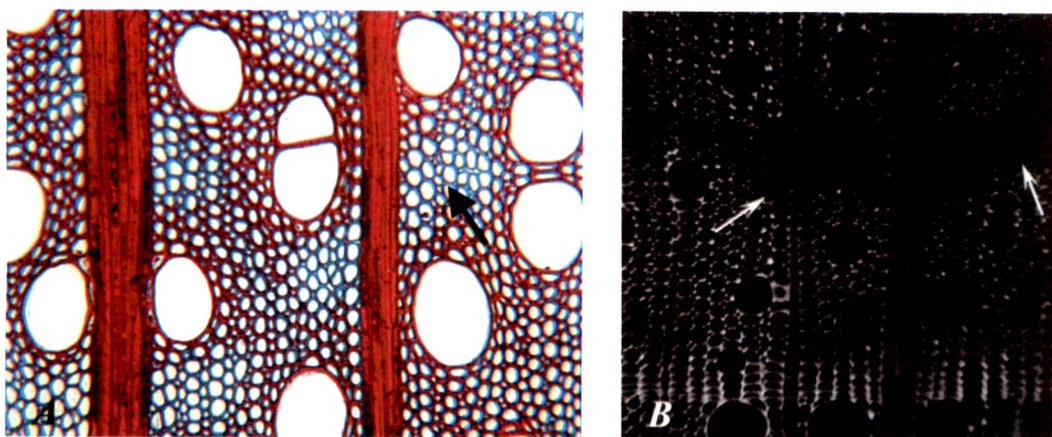


Fig. 3. Sugar maple. A) Stained with safranin O and astra blue in ethyl alcohol solution. Note the pattern of a positive reaction of cellulose (blue). These areas are composed of libriform fibers (arrow) that do not fluoresce under ultraviolet light. Observe in (B) darker areas (arrows) in which fluorescence is not observed using ultraviolet light. The areas in red in (A) are lignified; comparable areas show fluorescence in (B). A, $\times 100$; B, $\times 70$.

imum in the ultraviolet region (Lin 1992). We used a filter with excitation at 365 nm and barrier filter at 400 nm (Nikon Fluorescence Filter UV-1A). Because the fluorescence signal is proportional to the concentration of the substance being analyzed, our observations indicated low concentrations of lignin. These results were confirmed by the reaction with safranin O and astra blue dyes dissolved in ethyl alcohol.

Safranin O began to leach out during passage through a rinse with 30, 50, and 95% alcohol. When a counterstain like fast green FCF in 95% alcohol was applied, both the green stain and its solvent removed the safranin O from the unlignified tissues more rapidly than from lignin (Berlyn and Miksche 1976). This occurred in 15 sec in our study, though it may take as long as 2 min. It is possible that the same phenomenon occurs when safranin O is used with a counterstain like astra blue. We found that in 3 min safranin O was removed from the unlignified tissue in sugar and red maple and became distinctly blue. During this time, the astra blue did not remove safranin O from lignified tissues. In contrast, fast green removed safranin O from both lignified and unlignified tissues in 15 sec. It should be noted that the tissues (libriform fibers) that appeared to be unlignified in reality might contain syringyl instead of guaiacyl lignin. Further research is needed to determine the role of the ethyl alcohol in the differentiating reaction when it is used as a solvent for safranin O and astra blue.

Safranin O is highly selective for lignin and does not interfere with astra blue. Safranin O is applied until the section is strongly overstained, then differentially destained. On the other hand, astra blue shows affinity for cellulose and is incorporated into cellulose fibers only in the absence of lignin

(von Aufsess 1973). Safranin O stains lignin whether or not cellulose is present. This effect was observed in both sugar and red maple species, and the details were recorded on color micrographs. This double staining allows for simultaneous red-blue staining quickly and easily.

Srebotnik and Messner (1994) described the use of aqueous safranin O and aqueous astra blue as differential stains to assess the selectivity of fungal delignification. Their study proved that these dyes are ideally suited for screening fungal delignification, but their results did not depend on section thickness. We found, however, that sections 20 μm thick gave better results than thinner sections (e.g., 10 μm) because the color reaction is more vivid in thicker sections. Also, Rapp (1998) studied heavily decayed wood of beech and pine and found that the basic methine stain basacryl brilliant red BG (CI basic violet 16) mixed with astra blue FM in aqueous solution stained differently depending on the type of decay. In our study, the histochemical reaction also was different in the two types of wood examined. Normal wood was pink (Fig. 1 E-H for sugar maple, M-P for red maple) and tension wood was red (Fig. 1, A-D for sugar maple, I-L for red maple) indicating that normal wood is more lignified than tension wood.

Our present results indicate the feasibility of using safranin O and astra blue in ethyl alcohol solution for differentiating lignified from unlignified tissues, particularly for the identification of libriform fibers. Safranin O is one of the best stains to identify lignin, but using it with a blue counterstain like astra blue in ethyl alcohol is very effective because differentially colored results can be obtained. Our histochemical analysis and the use of UV illumination suggest that guaiacyl lignin is

almost absent in the libriform fibers (only present in the corners) and that these fibers occur in undulating bands in both sugar and red maple. Our method allows details to be retained on color micrographs. Remarkable differences in lignification clearly were observed around the vessels as well as in the latewood and earlywood of both species.

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An automated rotisserie system for processing Western blots

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Abstract

An apparatus to automate completely the processing of Western blots is described. The prototype is based on a popular rotisserie system design. The incubation chamber consists of an inner cylinder that rotates inside an outer cylinder (incubation chamber). The blot is contained in the inner cylinder. Two magnets are mounted at one end of the inner cylinder, and rotation of the inner cylinder is effected by two magnets mounted on a motor drive outside the incubation chamber. Movement of chemicals into and out of the incubation chamber is driven pneumatically, and the entire process is controlled by a computer. Processing a blot for chemiluminescent detection takes 7 h to complete without human intervention. The quality of the resulting image is comparable to or better than a blot using manual processing. In addition, the prototype is capable of re-collecting all three antisera for future use.

Key words: automation, programmable logic controller, rotisserie, Western blots

Immunoblotting, or Western blotting, is a widely used technique for detecting macromolecules such as protein (Towbin et al. 1979). This technique allows rapid identification of an antigen and determination of its apparent molecular weight. In general, proteins are separated using denaturing polyacrylamide gel (Laemmli 1970), and the separated proteins are adsorbed onto a blotting medium such as nitrocellulose. Processing the blot requires its incubation with primary antibodies, followed by secondary or tertiary antibodies that have been conjugated with detectable label. To ensure that the protein antigens on the blot are exposed evenly to the antibodies, the incubation and washing steps are done on an orbital shaker. The process normally takes a full working day to complete. In addition to the time required, the blots can be awkward to

handle and they often get torn during the process, especially when using nitrocellulose paper.

In recent years, a commercial rotisserie system has become available. The origin of this system is unclear. Thomas et al. (1988) first described the development of protein blots in rolling drums. In the early 1990s, units marketed under the names Navigator and Radian became available. Presumably, the rotisserie system was based on these earlier commercial systems used for Southern and Northern hybridization. Most rotisserie systems currently on the market use GL 45 thread size bottles of various lengths to carry the blot. Individual blots are sandwiched between nylon screens and inserted into the bottles. Hybridization solutions are added and the bottles are then rotated inside an oven. Not only does this system simplify changing chemicals in the bottles, but the volumes of chemicals used in the processing are reduced significantly. In addition, the system can process a number of blots (up to five) in the same bottle by sandwiching them between layers of nylon screens. The rotisserie system has become standard equipment in molecular biology labs, and many have adapted it for processing Western blots. Despite its simplicity, processing of Western blots on a

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rotisserie system still requires frequent trips to the processor to change reagents.

Diers (1994) described a semi-automated rotisserie system for processing Southern and Northern blots. Theoretically, the same system can be adapted for processing Western blots. Although the design reduces handling of hazardous chemicals, it does not reduce frequent visits to change chemicals. Prieur and Russo-Marie (1988) described the use of a PhastSystem (Amersham and Pharmacia Biotech, Piscataway, NJ) to automate Western blot processing. The price of a PhastSystem (approximately \$10 000) has prevented this system from becoming popular in the laboratory. In addition, the system only can process a 40 × 40 mm miniblott, and all the antibodies used in the processing are drained to waste.

We describe here a fully automated rotisserie system. The system moves chemicals in and out of an incubation chamber pneumatically, and it is capable of re-collecting the chemicals for future processing.

Materials and methods

Design of incubation chamber and reagent reservoirs

The entire incubation chamber was machined from Lucite and consists of two cylinders. The outer cylinder measures 187 mm in length, 90 mm O.D., 76 mm I.D., with a 6.4 mm wall (Fig. 1). The drive end of the outer cylinder houses all of the fluid and

pneumatic connections. This end is sealed with a circular 12.7 mm thick piece of Lucite that has the same diameter as the outer cylinder (Figs. 1 and 2). A circular depression, 63.5 mm diameter × 15.9 mm deep, was machined on this seal to receive the inner cylinder drive. Two openings on this seal are arranged at 120° to each other. One opening is the pneumatic connection, and the second opening is the inlet for reagents into the incubation chamber (Fig. 2). A single opening on the bottom of the incubation chamber is for the exit of reagents (Figs. 1 and 2). The open end of the outer cylinder is closed by a cap consisting of a 12.7 mm thick piece of Lucite with the same diameter as the outer cylinder. On the side facing the lip of the outer cylinder, the Lucite cap has a groove where an O-ring is fitted (Fig. 1).

The inner cylinder of the rotisserie measures 158 mm in length, 73 mm O.D., 67 mm I.D., and has a 2.3 mm wall (Fig. 1). At one end of the inner cylinder is a 25.4 mm wide cross bar housing the two drive magnets and a plastic bearing (Small Parats Inc., Miami Lakes, FL) (Fig. 2). The magnets are neodymium rare earth magnets (Rochester Magnet Company, Rochester, NY) and measure 6.3 mm in thickness and 19 mm in diameter. The magnets are completely sealed in the cross bar. At the other end of the cylinder, another cap houses a plastic bearing. Six 16 mm diameter holes spaced 60° apart are drilled into this cap (Fig. 2). During operation, the rotisserie is hung between the two Lucite rods (9.5 mm diameter × 38 mm length), one at the cap and the other on the sealed end of the outer cylinder (Fig. 1). The rotisserie is driven by two neodymium

Fig. 1. Schematic of the incubation chamber with the inner cylinder in place. During operation, a Teflon bearing (TW) must be inserted between the drive end of the cylinder and the drive end of the incubation chamber. The bearing consists of two Teflon washers fabricated from a 0.04 mm thick Teflon sheet. A 12.7 mm diameter Lucite rod with a UNF 1/4-28 port is glued to the bottom of the incubation chamber to serve as the reagent exit port.

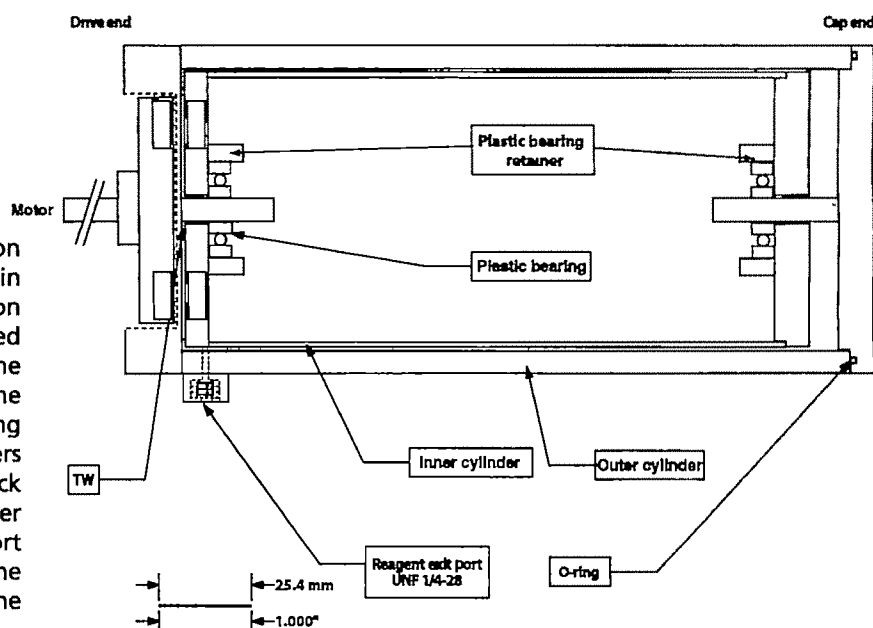
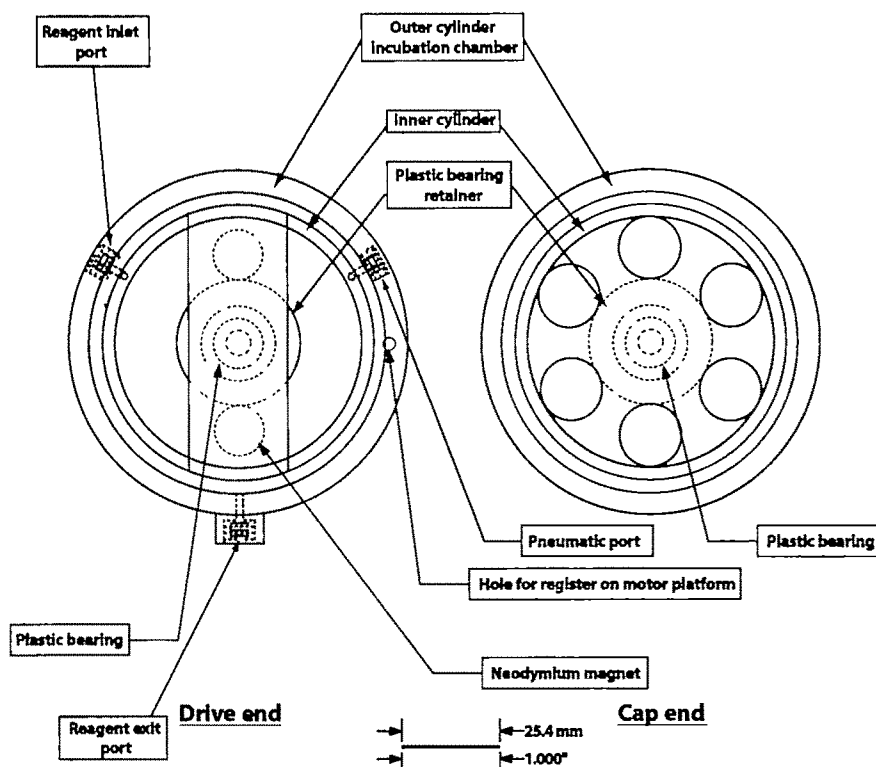


Fig. 2. Schematic of the inner cylinder at the drive end and the cap end. The positions of the neodymium magnets, the plastic bearings, and the plastic bearing retainers are shown in dotted lines. The plastic bearing retainer is glued directly on the acrylic cross bar of the inner cylinder (shown as two vertical lines in the figure). The reagent inlet port, pneumatic port, and reagent exit port, are arranged 120° apart and are shown in dashed lines. The reagent inlet port and the pneumatic port are machined directly into the seal at the drive end of the incubation chamber. The reagent exit port is machined into a 12.7 mm Lucite rod and glued onto the bottom of the incubation chamber near the drive end. A 4 × 8 mm (diameter × depth) hole is drilled on the seal of the incubation chamber at the drive end to register on the 4 mm rod protruding from the motor platform for quick orientation of the incubation chamber. The cap on the end of the inner cylinder where six 3/4 inch holes are drilled can be removed from the inner cylinder for insertion of the blots.



magnets mounted on a drive that fits into the depression at the sealed end of the incubation chamber. An AC motor running at 18 RPM drives the rotisserie.

All reagent bottles are made of Pyrex or equivalent with GL 45 thread. All glass surfaces that come into contact with antisera are treated with Sigmacote (Sigma Chemical Co., St. Louis, MO) and baked at 180° C for 3 h before use. Manifolds for all the reagent bottles were machined from 28.5 mm O.D. Teflon or Lucite rod. A representative construction of the reagent manifold is shown in Figure 3. Manifolds used on bottles to contain and re-collect antisera have three ports: two are fluid ports and one is a pneumatic port, and they are made of Teflon. Manifolds used on reagent bottles, such as washing buffer and blocking buffer, have two ports; one is a fluid port and the other is a pneumatic port. The manifolds were made of Lucite. All pneumatic and fluid valves were powered by 12 V DC. The pneumatic valves were Minimatix with UNF 1.4-28 ports (Clippard, Cincinnati, OH). Four three-way valves (Co-Palmer,

Rockford, IL) and one six-way valve (Bio-chem Valve, Boonton, NY) were used in the prototype (Fig. 4). The movement of fluid within the system is driven pneumatically using a nitrogen tank set at 15 psi. Figure 5 shows the set-up in the author's laboratory.

All fluid connections were made with 3.2 mm O.D. × 0.76 mm I.D. Teflon tubing using flangeless fittings (Upchurch Scientific, Oak Harbor, OR) or Teflon fittings with kel-F inserts (General Valve, Fairfield, NJ) with UNF1/4-28 threads. All pneumatic connections were made with 3.2 mm O.D. × 2.4 mm I.D. Tygon tubing with polypropylene barbed connectors (Valued Plastics, Fort Collins, CO). Pneumatic connections between the main pneumatic supply with the rest of the system were made with 3.2 mm O.D. copper tubing using Swagelok compression fittings (Swagelok, Solon, OH). Application of pressure at the reagent bottle or the incubation chamber causes the reagent to move from the reagent bottle to the incubation chamber, or from the incubation chamber back to the reagent bottle (Fig. 4).

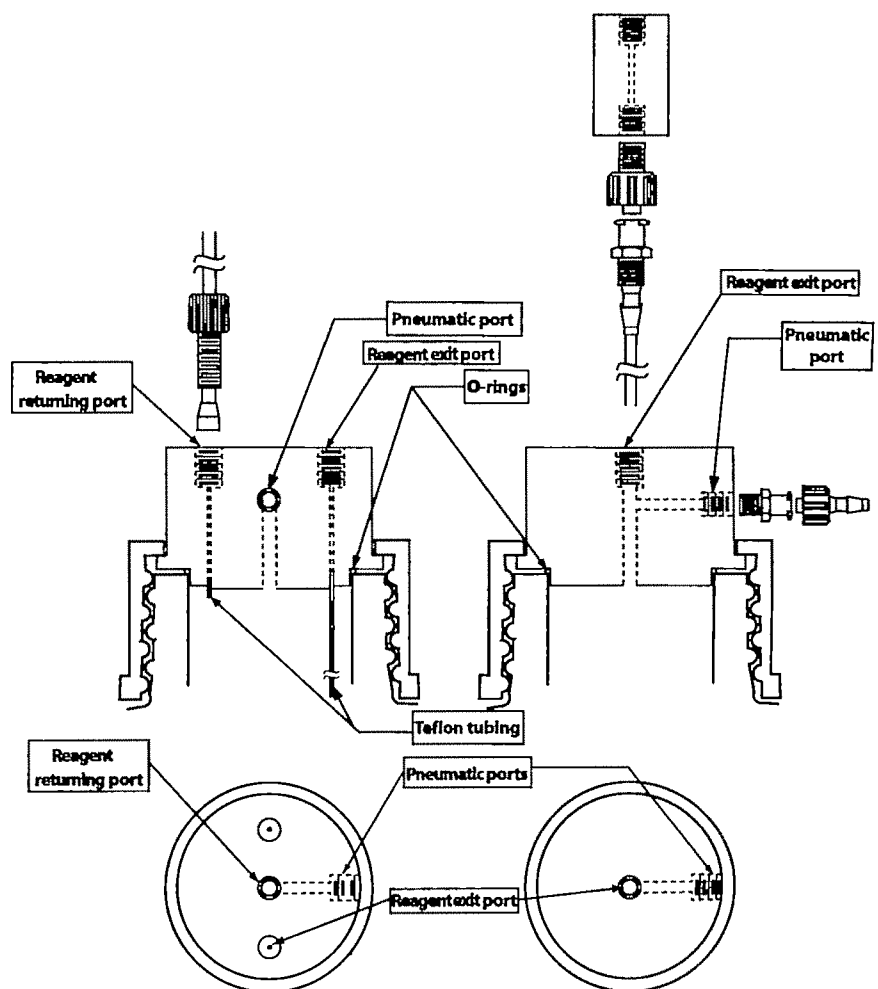


Fig. 3. Schematic of the side view (top) and top view (bottom) of the manifold for the reagent bottle with (A) and without (B) the reagent re-collection port. Only the neck of the bottle is shown. The manifold is held in place by screwing down the cap. A viton O-ring is fitted on the bottom of each manifold to create an airtight seal. The Teflon tubing used in (A) is pressed-fit into the manifold. Same type of fitting for pneumatic hook-up is used in both (A) and (B). All screw-in ports use UNF 1/4-28 thread.

A. Manifold with reagent re-collection port

B. Manifold without reagent re-collection port

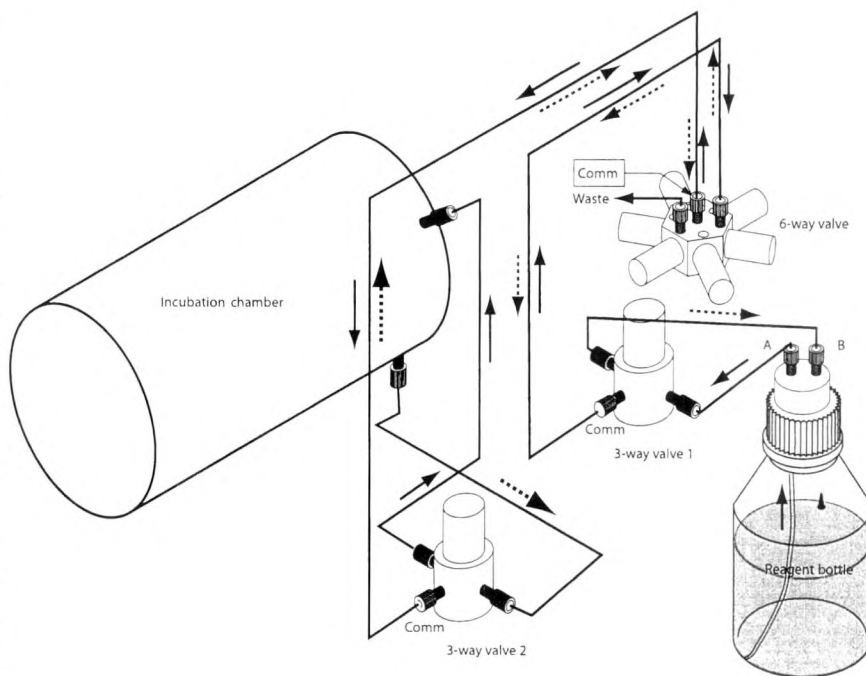
Programmable logic controller (PLC)

A programmable logic controller, Intelligent PLC (VIC Computer, H.K.), was used to interface the computer with all the fluid and pneumatic valves. Firmware (version 2.50K2) supplied by the PLC manufacturer was used in the communication protocol that was incorporated in the computer control software. The PLC communicates with the computer via the RS 232C interface at 9600 baud rate. The software used to control the system was written in BASICA (software available from author).

System operation

The prototype was used to process a Western blot by analyzing recombinant proteins expressed by a recombinant baculovirus AcBX-PBAN that contained a synthetic chimera gene encoding a bombyxin signal peptide sequence fused to a truncated pro-Hez-PBAN (pro-*Helicoverpa zea*-pheromone biosynthesis activating neuropeptide) sequence (Ma et al. 1998). Protein fractions obtained from size exclusion chromatography-fractionated hemolymph of cabbage looper larvae infected with AcBA-PBAN were separated using a buffer/gel

Fig. 4. The flow path of reagent into and out of the incubation chamber. The diagram is for illustration and is not drawn to scale. For clarity, only one reagent is shown and no pneumatic flow path is shown. There are no cross talks between flow paths in the diagram unless otherwise indicated. The common port (comm) on the fluid valves is connected to only one other port on the same valve at any time. When pressure is applied to the reagent bottle (inlet for pneumatic hook-up, not shown), flow of reagent into the incubation chamber is indicated by arrows with dashed lines. Reagents that would not be recollected, such as washing buffer and blocking buffer, follow a similar pathway except that the flow path of these reagents out of the incubation chamber is diverted to waste at the 6-way valve.



system described by Schagger and von Jagow (1987). Proteins were transferred onto nitrocellulose paper using a Bio-Rad TransBlot Cell (Bio-Rad, Hercules, CA). After transfer, the proteins on the blot were fixed by paraformaldehyde vapor at 70° C. Before loading the blot into the incubation chamber, the blot was sandwiched between two layers of nylon screen (Laboratory Product Sales, NY) and placed in a tray filled with PBS-Tw (phosphate buffered saline-Tween-20: 0.01 M sodium phosphate, 0.89% sodium chloride, 0.02%

potassium chloride, 0.05% Tween 20). The incubation chamber was tilted at about 2–5° on the motor platform and 40 ml PBS-Tw was added to the incubation chamber. The blot was rolled up and inserted into the rotisserie with the leading edge of the blot pointing toward the direction of rotation of the rotisserie. The cap of the incubation chamber was replaced and sealed by tightening the thumb-screw on the platform (Fig. 5). The motor on the platform was immediately turned on to relax the blot. The entire incubation chamber was then

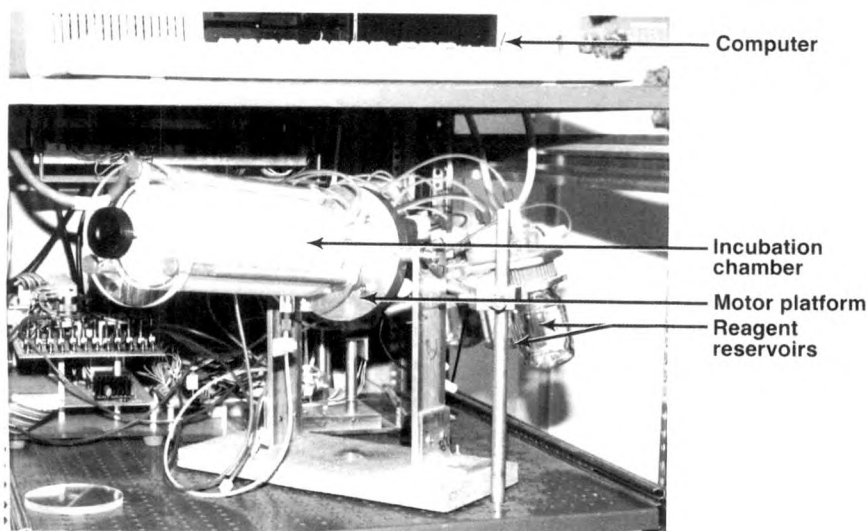
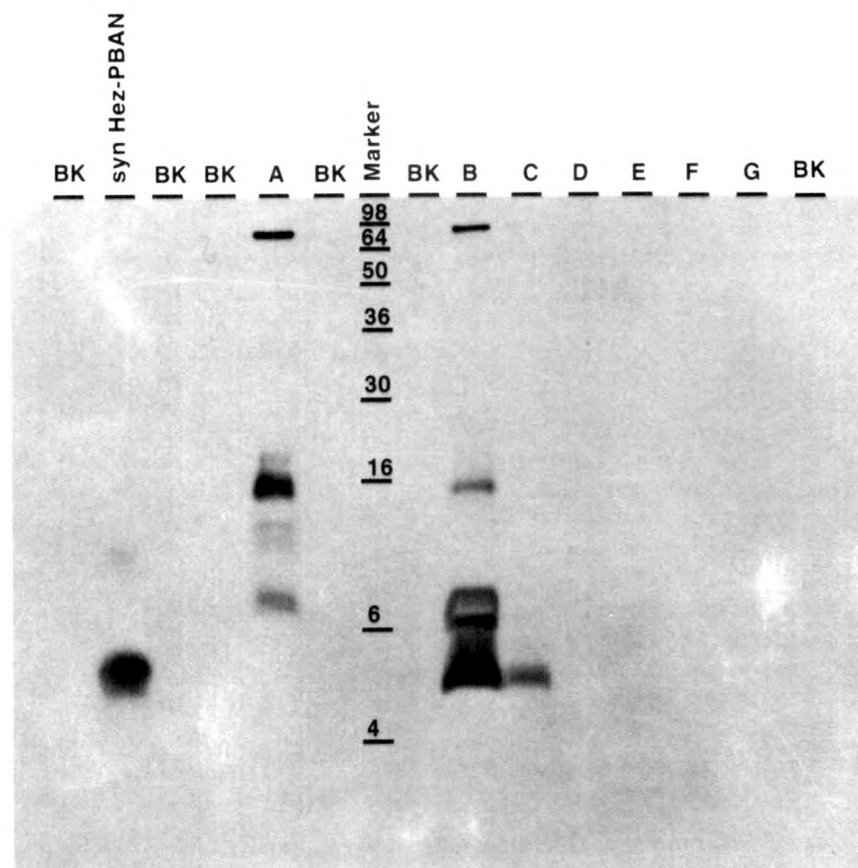


Fig. 5. The prototype set-up in our laboratory. The entire set-up is mounted on an instrument cabinet.

Fig. 6. Western blot processed by the prototype processor. Bars and numbers in the center of the blot indicate the positions and apparent molecular weights (kDa) of a SeeBlue pre-stained standard (Novex, San Diego, CA). Letters on top of the blot indicate size exclusion chromatography fractions of AcBX-PBAN-infected 4th instar *Trichoplusia ni* larvae hemolymph (Ma et al. 1998). The entire blot image is shown. BK, Blank.



lowered so that it was almost horizontal, and a programmed incubation/washing sequence as described by Lee and Scott (1992) was initiated. This programmed sequence was written in BASICA. In general, the program began with a query for the input of the incubation time for the primary, secondary and tertiary antisera, as well as the washing and blocking times. To test the prototype, we used a primary antiserum I-301-F that was raised against an insect neuropeptide PBAN (Ma and Roelofs 1995). Briefly, three layers of antisera were used, and the blot was incubated successively in 1:1000 I-301-F, 1:200 goat anti-rabbit antiserum (Calbiochemical, San Diego, CA) and 1:4000 peroxidase anti-peroxidase antiserum (Cappel, Durham, NC). Incubation of the blot with the primary antiserum was preceded by a 1 h incubation with a blocking buffer (PBS-Tw with 0.2% nonfat dry milk) followed by a 15 min PBS-Tw wash. The incubation time for each antiserum was 1 h, and each incubation was followed by three 15 min washes with washing buffer (PBS-Tw). At the end of the process, the blot was removed, and the signal was detected by a SuperSignal Chemiluminescent kit (Pierce, Rockford, IL) and recorded on X-ray film according to manufacturer's instructions.

Results

The protein blot processed by the rotisserie system described here is shown in Figure 6. Proteins that cross-react with antiserum I-301-F were localized in three fractions labeled A, B, and C (Fig. 6). The apparent molecular weight of these proteins ranges from 4.5 to 20 kDa. PBAN-immunoreactive bands that have the same mobility as synthetic Hez-PBAN were found in fractions B and C. In addition, there are other PBAN-immunoreactive bands in these fractions. These protein bands apparently represent post-translational processed fragments that contain the epitope FXPRL-NH₂ (X = Ser, Thr or Gly) that cross-react with antiserum I-301-F. The apparent molecular weight of these protein fragments ranges from 6 to 20 KDa. PBAN-immunoreactive proteins were not detected in fractions D-G. The results clearly show that the recombinant pro-Hex-PBAN was processed accurately because a protein band corresponding to the size of a synthetic PBAN sequence was found in the hemolymph of cabbage looper larvae infected with AcBX-PBAN.

The quality of the blot is equal to or better than a similar blot that was processed following a manual procedure (data not shown) using an orbital shaker.

The rotisserie system allows automated processing of the blot, and the antisera that were re-collected can be re-used at least three times without noticeable deterioration of signal (data not shown).

Discussion

The size of the blot used for testing the prototype was 140 × 150 mm. The prototype can accommodate slightly larger (up to 150 mm in height, the physical length of the inner cylinder) or smaller blots. When a smaller blot is processed, it is possible to push the blot into the rotisserie near the magnet and raise the entire incubation chamber by a few more degrees. In this case, the reagents settle at the lower end of the incubation chamber and less reagent is needed for processing (Thomas et al. 1998).

In the prototype described here, the reagent inlet and outlet of the incubation chamber are separated. This design eliminates the problems of the bubbles generated by pneumatically pumping reagents that contain detergents such as Tween-20. As an added security, however, an empty bottle was placed between the incubation chamber and the pneumatic valve to prevent condensation in the pneumatic lines from contaminating the pneumatic valves.

For the pumping system to work properly, the most important issue is to maintain an airtight system. This is accomplished by clamping the incubation chamber onto the motor platform. Tolerance of the manifolds is not critical as long as a viton O-ring is inserted in the bottom of the manifold to provide a good seal between the manifold and the reagent bottle. After processing over 50 blots, we have not experienced any leaks that require shutting down the system.

In the prototype, all the pneumatic valves were grouped together and mounted on top of the power supply board. To facilitate manual control of all the valves, a three-position switch was installed to operate each valve. These manual switches are not necessary for normal operation of the system. They are helpful, however, for method development and for emergency purposes so that some parts of the system can be shut down if problems occur during program execution.

In general, it takes about 7 h to process a blot. Up to five 140 × 150 mm blots can be processed in one run. The run time includes a 30 min post-run clean up. A post-run clean up was performed by replacing all the reagent bottles with distilled water. This is necessary to prevent buffer from drying inside the fluid valves and causing blockage. The post-run clean up washing sequence took 15 min to

complete. In our lab, a different set of bottles filled with distilled water were prepared before the commencement of the run so that one needs only to replace the bottle at the end of the run. This allows the operator to concentrate on processing the blot for signal detection while the clean up sequence is underway. Normally, if the same antiserum is used for further blot processing, one can reload the system (adding the washing buffer and blocking buffer) and empty the waste. Thus, the system can be operated around the clock without intervention.

During the writing of this report, we became aware of a commercial apparatus for automated processing of Western blots that recently became available from Stovall (Greensboro, NC). The basic commercial unit costs \$4000, and approximately \$4750 with some of the necessary accessories. The major differences in the design of the commercial system and the prototype described here is that the commercial system uses an orbital shaker to agitate the blot, and a vacuum was used to move the reagents. The author does not have experience with this commercial unit, but it does not appear to have the capability to re-collect antiserum.

In the prototype described here, the inner cylinder is rotated on two plastic bearings. The original design with the bearing in place was to eliminate as much friction as possible to provide a smooth rotation of the inner cylinder. In practice, these bearings are not necessary. Instead, a Teflon sleeve can be used to replace the bearing, and can be fabricated easily in the shop. A Teflon bearing made of two 0.04 mm thick Teflon washers is needed, however, at the end of the inner cylinder near the drive mechanism to compensate for the high magnetic force generated by the neodymium magnets of the drive mechanism.

Using the system described here, we routinely used 60 ml reagent, although as little as 20 ml would keep a 140 × 140 mm blot wet in the incubation chamber. This is important if the amount of primary antiserum, such as a monoclonal antibody, is limited. Another advantage of our system is that all three antisera can be re-collected and re-used. It is possible to reconfigure the system so that two different types of primary antisera can be used simultaneously on two separate sets of blots in two separate incubation chambers. The PLC used in our prototype contains 12 outputs. Only ten outputs were used in the design of the prototype. There is also a stepping motor driving circuit on the PLC that can drive a three or four phase stepping motor. To make full use of all the outputs on the PLC board, one can use the remaining two outputs to control a separate set of pneumatic and fluid valves.

The stepping motor driving circuit can be used to control a stepping motor to switch the flow path to two separate incubation chambers. Theoretically, this could allow two different primary antisera to be used in two incubation chambers to process two blots.

The materials used in the construction of the system described in this paper cost about \$1200 (excluding the computer). Our computer is an IBM PC that was retrieved from departmental storage. If one considers running a blot once every two days, the time saved in six months is worth the construction of the described system in this paper. It took the author two full days in the shop to build the incubation chamber and all the manifolds, and a full day to assemble the system. Therefore, the system described in this paper presents an economical solution for laboratories that routinely process a large number of Western blots.

Acknowledgments

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Selective fluorescence of zymogen granules of pancreatic acinar cells stained with hematoxylin and eosin

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Abstract

Following staining with hematoxylin and eosin Y, paraffin sections of mouse pancreas were examined by transmitted light, epifluorescence and confocal laser scanning microscopy. Light microscopy revealed that the nuclei of pancreatic acinar cells were located basally, while the apices of the cells appeared eosinophilic, although the secretory granules were difficult to visualize. Under violet-blue light excitation, the zymogen granules at the apices of the acinar cells showed strong yellowish fluorescence; the other part of the cytoplasm was only faintly fluorescent and the nuclei and the supporting tissues were nonfluorescent. Confocal laser scanning microscopy resulted in clear pictures of the zymogen granules and their distribution within the cell. The fluorescent emission of zymogen granules was certainly the result of eosin Y staining, because hematoxylin is not a fluorochrome and the zymogen granules are not autofluorescent. Staining with eosin Y alone, however, did not result in clear fluorescent images of zymogen granules or any other cellular structures. Our observation shows that the fluorescence emission of eosin Y allows easy and precise recognition of zymogen granules of pancreatic cells.

Key words: acinar cells, eosin Y, fluorescence, pancreas, zymogen granules

Pancreatic acini are irregular clusters of pyramidal cells, the apices of which surround a minute central lumen representing terminal ends of a duct system. The secretory granules of pancreatic acinar cells contain a wide range of proenzymes (zymogens) including amylase, lipase, trypsin, carboxypeptidase and nucleases (Young and Heath 2000). Zymogen granules of pancreatic acinar cells can be demonstrated by immunofluorescence techniques and electronic microscopy (Oomori et al. 1994, Young and Heath 2000); however, simpler and more reliable methods are also of interest, because pancreatic acinar cells have long been a favored example of protein secreting cells; their ultrastructure and biochemistry have probably been studied

more intensively than any other glandular cells. We report here that hematoxylin and eosin Y (H & E) staining provides a simple and reliable means of visualizing the zymogen granules of pancreatic acinar cells by fluorescence microscopy.

Materials and methods

A standard protocol was employed to prepare paraffin sections used in this study. In brief, mouse pancreases were fixed with 10% formalin solution followed by paraffin embedment. Then 5–8 μ m sections were dewaxed, rehydrated, and stained with Mayer's hemalum for 5 min, washed briefly in running tap water, then counterstained in 0.5% aqueous eosin Y (C.I. 45380; Sigma-Aldrich, St. Louis, MO, USA). After two washes, sections were dehydrated and mounted in Merckoglass (Merk, Germany). Unstained sections were also mounted and included as controls. Observation and photography were performed using a Leica epifluorescence photomicroscope (DLMB LPS30, Heidelberg,

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Germany). Confocal microscopy was performed using a Leica confocal laser scanning microscope (TCS SP2) using an argon laser with an excitation wavelength of 488 nm and an emission range of 490–550 nm. The microscope was also equipped with dry and oil immersion objective lenses, and the specimens were evaluated and photographed with filter set for green. Data were analyzed using the TCS NT software. Digital images were transferred to a PC computer equipped with ACDSee program and printed on an ink jet printer (Epson Photo 710).

Results and discussion

As expected, light microscopy showed that the nuclei of pancreatic acinar cells were basophilic (stained blue) and located basally; the apical half of the cytoplasm appeared eosinophilic (pink-red), although the secretory granules were difficult to visualize (Fig. 1A). Under violet-blue (436 nm) light excitation, the basal part of the cytoplasm of the acinar cells showed a background level of green fluorescence. In contrast, a bright yellowish emission was observed in the apices of the cells (Fig. 1B). The nuclei, capillaries, collagenous septa between

the lobes, and the supporting tissue separating adjacent acini were all nonfluorescent. Confocal laser scanning microscopy resulted in clear pictures of zymogen granules and their distribution within the cell (Fig. 1, C–E). The central lumen of the acinus was nonfluorescent, but could be identified by the fluorescent granules concentrated in the apices of the surrounding acinar cells (Fig. 1D). Unstained paraffin sections of mouse pancreas showed only a background level of gray-green fluorescence (data not shown). Unfixed sections also were examined to ensure that the pancreatic zymogen granules had no detectable autofluorescence. Because hematoxylin is not a fluorochrome, it can be concluded that the selective fluorescence of zymogen granules of pancreatic acinar cells was due to eosin Y staining. It is of interest to note, however, that staining with eosin Y alone did not produce clear fluorescent images of zymogen granules or any other cellular structures of pancreatic acinar cells under the fluorescence microscope (Fig. 1F).

Eosin Y is an acidic dye that stains basic structures red or pink. In addition, eosin Y is a fluorochrome compatible with excitation by an argon ion laser at 488 nm with emission at 520 nm

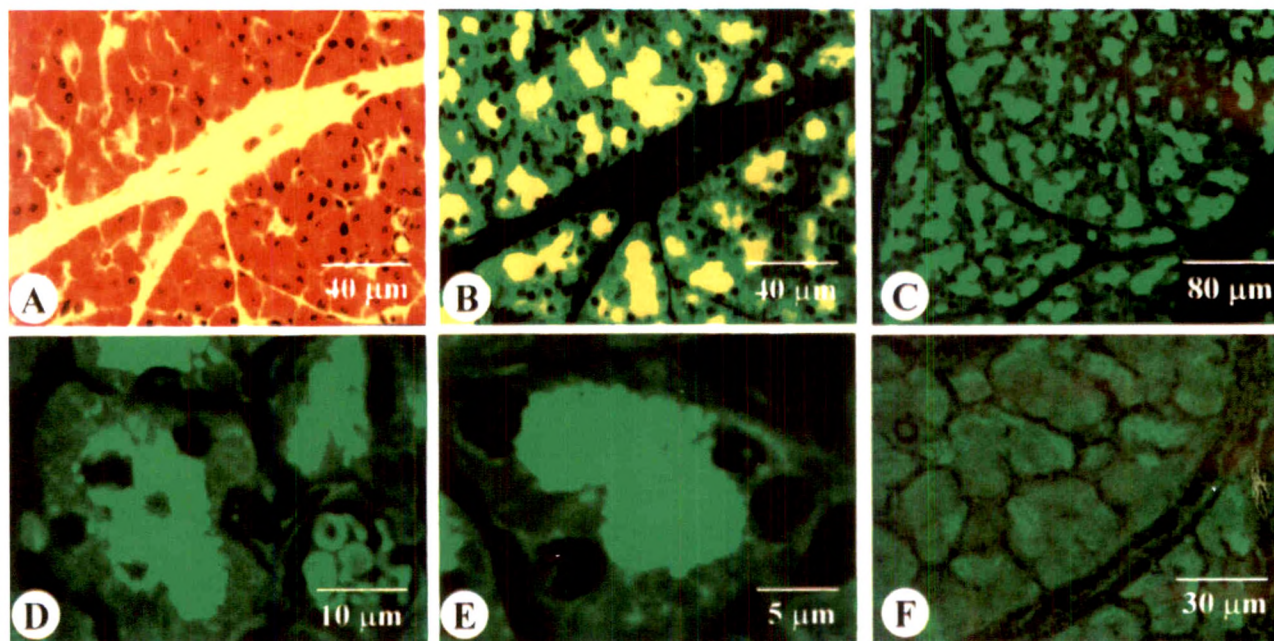


Fig. 1. A) Light microscopy of H & E stained paraffin section of mouse pancreas showing that the nuclei of pancreatic acinar cells are located basally while the apices of the cells appear eosinophilic. Note that the zymogen granules are barely recognizable. Original magnification $\times 400$. B) Epifluorescence microscopy showing the same field as in (A). Strong green fluorescence is observed in the apices of the pancreatic acinar cells. The nuclei, capillaries, collagenous septa between the lobes and the inconspicuous supporting tissue separating adjacent acini are nonfluorescent. C–E) Confocal laser scanning microscopy showing fluorescent zymogen granules and their distribution in the apices of the cells at different magnifications ($\times 200$; $\times 1600$; $\times 3200$, respectively). The nonfluorescent central lumen is revealed by the surrounding fluorescent granules in the apices of the acinar cells (D). F) A pancreatic tissue section stained with eosin Y alone, then evaluated using confocal laser scanning microscopy.

(Seybold et al. 1969). A selective fluorescence reaction after eosin Y staining has been observed in elastic fibers (de Carvalho and Taboga 1996), rat embryo sections (Apgar et al. 1998) and certain other structures in grasshoppers and mice (Espada et al. 1993). Our observation shows for the first time that eosin Y also allows easy and precise recognition of the secretory granules of pancreatic acinar cells by fluorescence microscopy.

As an acidic (anionic) dye, eosin Y tends to bind to basic rather than acidic structures. Several groups of researchers have demonstrated that pancreatic zymogen granules are acidic within the cell (Niederau et al. 1986, de Lisle et al. 1988), which raises the question about why the eosin Y was able to stain the zymogen granules of pancreatic acinar cells. It is reasonable to presume that the zymogen granules were only faintly stained by eosin Y. De Carvalho and Taboga (1996) noted that fluorescent structures in H & E stained sections were all faintly stained by eosin and intensely eosinophilic structures usually showed very little fluorescence, probably resulting from "concentration quenching".

H & E staining is the most commonly used technique in histology and routine pathology. The advantages of using H & E staining to reveal fluorescent structures reside not only in its simplicity, sensitivity and reliability, but also in the possibility of studying the structures in file materials without further sectioning and staining.

Acknowledgments

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Editor's note

With the paper that follows we inaugurate a new series entitled "Names you know, but don't know why." Users of biological dyes and stains likely are familiar with the names of persons associated with certain dyes, stains and procedures. We suspect, however, that most readers are unfamiliar with the contributions that put these names into the vocabulary of histochemistry and histotechnology. We thought it interesting to investigate these figures from the past and to report our findings in a series of papers beginning with Dr. Kasten's paper about Dr. Ralph Lillie. The format will be to introduce the stain or procedure associated with an historical figure and to follow this with a short biography and a few salient references. I look forward to reading these intriguing bits of the history of our field, and I trust our readers will enjoy them as well.

Ralph D. Lillie: eminent histochemist and pathologist

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It is fitting that this journal's new series of vignettes of great figures in the field of histochemistry and the stains associated with them begins with Ralph D. Lillie, an internationally recognized pioneer and innovator. He is regarded as one of the world's leading 20th century authorities in the field—a master magician of histochemistry.

Let us recount the highlights of the career of this research scientist and physician. He was born August 1, 1896 and died October 5, 1979. Listed under "References" are two of Lillie's books (Lillie 1977, Lillie and Fullmer 1976) and a reference to a complete chapter of Lillie's life and accomplishments that includes a bibliography of his 337 research publications, eight photographs, and a listing of ten other articles about and obituaries for Lillie written by his associates and former students (Kasten 1983).

During the late 1950s and 1960s, I became personally acquainted with Dr. Lillie at annual American Histochemical Society meetings and at international meetings in Frankfurt, Hamburg and New York. Little did I know at the time that he was quietly looking me over as a potential colleague at Louisiana State University Medical School in New Orleans. In 1969, he heard that the Anatomy Department was looking for a professor and whispered my name in the ear of the department head, Dr. Melvin Hess. In January of 1970, I was pleased to join Lillie as a fellow faculty member at the school.

It may come as a surprise to modern-day histochemists to learn that Ralph Lillie was a prankster in the public schools of San Francisco and Monterey, California, and that he was referred to as "Peck's bad boy." He later graduated from Stanford University with an A.B. in 1917 and an M.D. in 1920. His bent toward research was already

evident in medical school, where he published his first two papers in the fields of gross anatomy and blood cytology. During his research career, he rarely published purely clinical papers. He was introduced to experimental pathology by William Ophuls, a noted pathologist at Stanford and San Francisco General Hospital. Ophuls himself was a student of Johannes Orth, who in turn had studied under Rudolf Virchow. Thus we can trace Lillie's professional genealogy back to the "Pope of 19th Century Pathology."

Lillie joined the Public Health Service in 1920, first working in hospitals, quarantine stations, and immigrant points on the east and west coasts. In 1925, he was assigned to the Hygienic Laboratory in Washington, DC, the precursor of the National Institutes of Health. He became Chief of the Laboratory of Pathology and Histochemistry in 1948, and served there until he retired in 1960. For the first 15 years of his career, Lillie was involved in experimental pathology, making important contributions in nutritional pathology, infectious diseases, and pharmacological toxicology.

Lillie's research concerning biological staining first surfaced in 1928. At this time, his brief foray into the field of staining led to two papers on the Gram stain, one on calcium and one on mucus detection by dyes. It is interesting that the latter two brief contributions appeared in German in the respected *Zeitschrift fuer Wissenschaftliche Mikroskopie*. They would be his only papers published in German, a language that he enjoyed reading.

In 1932, and again in 1936, Lillie published with Pasternak on Romanowski staining of tissues with buffers. Lillie's early interest in the classical blood stain heralded his important work on methylene blue and other thiazine dyes, which he carried out intensively in the 1940s. In 1938, Lillie reported on hemosiderin and on the Masson trichrome modification of Mallory's connective tissue stain.

Lillie joined the Biological Stain Commission as a Trustee in 1940, and served for 39 years. It may be



Fig. 1. Dr. Ralph D. Lillie examining a slide at Louisiana State University Medical School in November, 1971. The monocular microscope was a favored relic "on loan" from Lillie's days at the NIH. As Research Professor of Pathology in New Orleans at the age of 75, and already retired from the NIH for 11 years, Lillie was still actively carrying out histochemical research.

recalled that the Commission had been in existence more than 15 years as a result of the consummate work of Harold J. Conn. Lillie was able to contribute his expertise to the work of purifying, characterizing, and applying biological stains for standardization. When the onset of World War II resulted in the unavailability of popular German dyes for hematology, Lillie busied himself with developing satisfactory blood and malaria stains based on the thiazine dyes. In 1940 and 1943, Lillie and his associates described the preparation of an acceptable Giemsa stain from American-derived azures and methylene blue. He considered this work to be one of his major accomplishments. During this wartime period, Lillie investigated polychrome methylene blue. About 35 years later, during the last two years of his life, Lillie returned to the problem of using dichromate to control the oxidation of blood stains with low azure B eosinates.

We should recognize that Dr. Lillie was self-trained in the methodology of organic chemistry and became proficient in the synthesis and purification of dyes and their chemical mordants. With a twinkle in his eyes, the master histochemist would eagerly tell visitors to his laboratory that he was a self-taught chemist. The chemistry books surrounding him showed the marks of much use. As an

affirmation of these practical abilities, the American Institute of Chemists named him a Fellow in 1969.

Beginning in the 1940s, Lillie devoted himself full-time to staining technics and histochemistry. A prodigious worker, he produced with various associates as many as 15 papers a year. Modern histochemists and histotechnologists who are unfamiliar with Lillie's work may be surprised to learn the breadth of his interests and accomplishments. He wrote articles on embedding paraffin blocks in the vacuum oven, histologic mounting media, lipid stains, bone decalcification, myelin, diastase to digest glycogen, dried egg white as a section adhesive, connective tissue, reliable hematoxylin stains, detection and characterization of pigments encountered in pathology (hemosiderin, ceroid, acid formaldehyde hematin), employment of Schiff's reagent after periodate (PAS) to detect reticulum, glycogen and mucins, a cold Schiff recipe, Feulgen reaction to detect parasites in tissues, performic and peracetic acid-Schiff reactions for cystine and cysteine, methylation technics, aldehyde blocking reactions, staining of melanin, elastic tissues, lipofuscins, amino acids, pyrroles, zymogen granules, collagens, enterochromaffin substance, acylation of end groups, histochemical reactions of bile pigments, orcein and azo dye staining of elastin,

examination of the Sakaguchi reaction for arginine, iron tannin staining, azo coupling reactions, staining of mast cells, metal mordant staining of nuclei and staining of basic proteins by alkaline Biebrich scarlet.

Lillie's legacy rests on more than his voluminous bibliography of research works. He left two valuable books for his successors in the field. After Dr. Frank Mallory died in 1941, and it became clear that Mallory's 1938 book on Pathological Technic would not reappear as a new edition by another author, Lillie began his own book to fill the void. His volume, *Histopathologic Technic*, appeared in 1948, and became so successful that it appeared in three further editions under a revised and modernized title, *Histopathologic Technic and Practical Histochemistry*. The new editions reflected overwhelming emphasis on histopathology and histochemical methodology, much of which was contributed by Lillie's own research. The final edition in 1976, co-authored by Dr. Harold M. Fullmer, reflected Lillie's thoroughness in tabulating data and his thorough knowledge of the older literature.

The second book associated with Lillie's name is the ninth edition of *H. J. Conn's Biological Stains* (1977). H. J. Conn first wrote *Biological Stains* in 1925 to be a reliable resource encyclopedia of dyes and their application to standardized biological staining. As the principal book published by the Biological Stain Commission, it went through eight editions under Conn's name until 1969, when the author was 83 years old. Soon afterward, Conn turned over the writing of the next edition to Lillie. This was an amiable transfer and led to the publication of the well-known ninth edition published under Lillie's name. The updating and inclusion of new material reflected Lillie's imprint on a standard reference in the field. A revised tenth edition has recently been published under the editorship of R.W. Horobin and J.A. Kiernan.

Lillie played a major role in the founding of the Histochemical Society in 1950. He served as the

Society Secretary from its inception to 1955, and became President in 1957. In 1953, he became the first editor-in-chief of the Society's *Journal of Histochemistry and Cytochemistry*. He held this vital position for 11 years.

Dr. Lillie's numerous honors included being named Honorary Vice-President of the Third and Fourth International Congresses for Histochemistry and Cytochemistry in New York (1968) and Kyoto (1972). In 1971, when the Histochemical Society held its annual meeting in New Orleans, the opening day, November 16, was proclaimed by Louisiana State University School of Medicine, Tulane Medical School, and the Society as "Professor Ralph D. Lillie Day" and treated as a "Festschrift" in honor of Lillie's 75th birthday. At the meeting, Dr. Lillie was named Honorary President of the Histochemical Society and given an official letter of greeting from Mayor Moon Landrieu, plus a book of congratulatory messages from colleagues around the world.

Upon retirement from the NIH in 1960, Lillie began a further career at the Louisiana State University School of Medicine in New Orleans. At the age of 64, he was given a laboratory and office where he worked vigorously for 19 more years. His extraordinary productivity in the face of advancing years and failing eyesight is a tribute to a keen mind and exceptional tenacity. Following his death from a stroke in 1979, the annual meeting of the Histochemical Society in New Orleans was dedicated to his memory.

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Notes and queries

This section contains anonymous questions and signed, peer-reviewed answers to questions on topics within the scope of the journal. Send questions or answers by mail to: Dr. JA Kiernan, Department of Anatomy and Cell Biology, The University of Western Ontario, London, Canada N6A 5C1, or by email to kiernan@uwo.ca.

Charcot-Leyden crystals

What are Charcot-Leyden crystals and how are they detected in a smear or cytopsin preparation from nasal washings?

Charcot-Leyden crystals are acidophilic, needle shaped bodies thought to be derived from the cytoplasmic granules of eosinophils. They are associated with chronic respiratory disease, particularly asthma (Lynch et al. 1969). They may also be found in tissues carrying a heavy parasitic infestation (Sandritter and Wartmann 1967).

Charcot-Leyden crystals may be stained by eosin or other acid dyes (Luna 1968) at any pH, even from neutral or alkaline solutions that fail to stain collagen and most kinds of cytoplasm. In an ordinary H & E preparation with bright field illumination, they are red and somewhat opaque.

In addition, Charcot-Leyden crystals are birefringent: bright on a dark background when examined in a polarizing microscope with crossed polars (Culling et al. 1995).

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Wavy sections

Although they flatten normally on the water bath, paraffin sections of skin are not lying flat on the slides. They adhere well and stain evenly, but look like waves. The microscope must be refocused as the section is moved. How can this artifact be prevented?

I have found that paraffin sections of skin stick well to albuminized slides if they are left on the warming tray for 48–72 h before staining. For all sections of skin, it helps to leave the slide on the warming tray for several hours after mounting with a 50 g weight on the coverslip.

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Apparent dirt from air drying

Some sections looked fine when they were wet after staining with an immunohistochemical method. After coverslipping, the sections were black and granular all over, but there wasn't any dirt on other parts of the slides. What went wrong, and how can it be prevented or remedied?

This artifact is caused by air drying the sections before applying the mounting medium. Tiny pockets of air are trapped within the section and they impede the passage of light. Make sure that sections are wet with xylene before putting a resinous mountant on them, or wet with water before using an aqueous medium. You should be able to resuscitate your sections by soaking in xylene, removing the coverslips, and replacing them without allowing air drying to occur.

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Fluorescent fat stains

Is there a fluorescent stain for fat cells?

Three come to mind immediately and there are certainly more.

1. An acridine dye called phosphine (CI 46045, basic orange 15), which has many synonyms, was introduced for this purpose in the 1940s. The fluorescent emission was described as silvery blue-green (Volk and Popper 1944). It has never been a popular stain, but it is still used occasionally (Horobin 2002).
2. A solution of 3,4-benzpyrene in water (together with some caffeine to bring it into solution) provides a fluorochrome for lipids of all types (Pearse 1985). The reagent is carcinogenic, so this method is not popular.
3. Nile red, an oxazone, can be made in the laboratory by a simple procedure with the chloride of Nile blue (CI 51180, basic blue 12) as the starting material (Greenspan and Fowler 1985). Alternatively, it may be bought ready-made for about 40 times the cost of Nile blue. Nile red is used as an approximately 0.5 µg/ml solution (made by 1000-fold dilution of a 0.5 mg/ml stock solution in acetone into a 75:25 glycerol:water mixture). A drop of the dilute solution is placed on the section under a coverslip, and the preparation is examined 5 min later. The emission from fat is yellow-orange; from phospholipids, red (Fowler and Greenspan 1985). Nile red is fluorescent only when present in hydrophobic objects such as lipid droplets or membranes.

Of these, the Nile red method is probably the one of choice.

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Spray-on fixatives for cells and hair

Please provide a recipe for making up a spray fixative for cells on slides. The labels on commercial solutions show the ingredients, but not how much of each. I'm told that hair spray is suitable. Is it?

Don't use hair spray. Its use in cytology was discredited long ago. The composition is usually undefined. If by chance you're using a hair spray that produces results you like, the manufacturer can change the composition without your knowing it. The new formula may produce different results.

To make spray fixative that equals commercially available ones, simply dissolve 20 g Carbowax 1450 in 95% ethanol up to 1000 ml. Denatured alcohol works equally well. You may want to dilute it to 95% concentration, but it won't make a visibly detectable difference in cytomorphology. (Denatured alcohol is 9 parts ethanol, 0.5 parts isopropanol, and 0.5 parts methanol.)

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Some 20 years ago I bought 24 random cheap hair sprays and conducted a trial. All did well, and I could not readily tell one from another in terms of results. A problem, however, is that the spray comes out with such force that if held too close, the cells could be on the far wall before one realizes, and if held at an ideal distance, it also fixes half the bench and everything on it.

The real point, of course, is that the formula given by Gary (above) is so easy and cheap to make and put into a pump spray that there is no need to look elsewhere.

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Coverslipping resin sections without bubbles

How do you avoid bubbles in the mounting medium when putting coverslips on sections of plastic-embedded tissue that are air dried onto glass slides?

It usually helps to thin the mounting medium with a little xylene. For glycolmethacrylate (GMA) sections, put a drop of mounting medium on the edge of the slide, just below the section, and mount the coverslip by lowering it onto the drop, with slight pressure to spread the mountant under the coverglass and over the section. For polymethylmethacrylate (PMMA) sections, the solvent in the mounting medium will crack the PMMA, giving an ugly look to your preparations. Instead of making a permanent mount, place a coverslip on top of the dry section. The glass will behave optically as a mounting medium with the ideal thickness and refractive index for use with high power microscope objectives. You will be able to obtain sharply focused photomicrographs without bubbles.

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Slide preparations for methylmethacrylate (MMA) and glycolmethacrylate (GMA) sections prior to coverslipping are different. For many years I have used Eukitt mounting medium (Calibrated Instruments, Hawthorne, NY) exclusively. MMA sections are deplasticized prior to staining: dehydrate with graded alcohols to absolute ethanol and clear with xylene (or a substitute) prior to coverslipping, as with paraffin sections. GMA resin cannot be dissolved. The sections are stained, dehydrated with graded alcohols to absolute ethanol, then completely air dried. Pass the air dried slides through two changes of xylene before coverslipping.

Depending on the thickness of the mounting medium, bubbles can form under the coverslip. If the mounting medium is too thick, very small bubbles may form. Shrinkage of the mounting medium occurs as its solvent evaporates. Consequently, if the mounting medium is too thin, bubbles may intrude under the edges of the coverslip after the mounting medium has “dried”.

Bubbles also will form if a bit of plastic embedding medium is sticking up above the level of the section. This can be seen if you look for it, and scraped off with forceps before coverslipping.

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I usually let my GMA sections dry if they come from water, then dip the slide quickly in xylene before coverslipping to help the mounting medium spread.

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Book review

Microscopy, Immunohistochemistry, and Antigen Retrieval Methods for Light and Electron Microscopy, MA Hayat. Kluwer Academic/Plenum Publishers, New York, Boston, Dordrecht, London, Moscow, 2002

As described in the preface of this book, the author, Professor Hayat, has the goal of introducing recent developments in the histochemical approach to microscopy, particularly immunohistochemistry. The heat-induced antigen retrieval (AR) technique is the major topic, and accounts for more than half of the book. The author summarizes the major applications of this valuable technique very well, based on recent publications as well as personal observations. Professor Hayat highlights several key issues in the application of AR technique such as temperature and time of heating, and pH of the AR solution, which are major factors that influence the effectiveness of the AR treatment. The author also discusses "test battery approach" as a convenient and rapid means to optimize these important factors, and emphasizes that retrieval of different epitopes may require different specific retrieval conditions.

The author also presents a rather concise review of several new techniques of molecular biology in the first chapter, essentially introducing the field of "molecular morphology", although the author does not use this term. For example, data are presented for several commonly used markers, including MIB-1, p53, ER, and HER-2, to illustrate the unique

advantages of a combined histological and immunohistochemical approach that has been modernized as "molecular morphology".

As a whole, this book provides useful data and guidance in the practical application of the histochemical, immunohistochemical and antigen retrieval methods for the diagnostic and research pathologist. As the title suggests, electron microscopy is covered, but while there are segments dealing with this, particularly with regard to fixation, the major focus is on light microscopy.

Specific areas of diagnostic application that are discussed include proliferation markers, estrogen receptors and BER2, with chapters on each. These are detailed, helpful and well referenced with useful sections on image analysis and quantitation; however, other vast areas of practically useful immunohistochemical applications are untouched.

Overall the book is slim (300 pages) and should find a place on the reference shelf of any self-respecting immunohistochemistry lab. That it will not earn a prominent place, however, is largely due to the disappointing quality of the pictures, with the exception of some rare color plates.

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Stains recently certified

The batches of dyes approved since those listed in the last issue of this journal are listed in the table below. Certification does not imply approval for medicinal use.

Stains certified from June 1, 2002 through July 31, 2002*

<i>Name of dye</i>	<i>Certification number</i>	<i>Manufacturer and/or vendor</i>	<i>Date approved</i>
Alizarin red S	AcAr-17	Aldrich Chemical Co.	July 11, 2002
Azure A	AcAz-34	Aldrich Chemical Co.	July 11, 2002
Brilliant green	AcBg-17	Aldrich Chemical Co.	June 4, 2002
Cresyl violet acetate	AcW-20	Aldrich Chemical Co.	July 11, 2002
Erythrosin B	AcEr-12	Aldrich Chemical Co.	June 4, 2002
Hematoxylin	DcH-19	Dudley Chemical Co.	June 4, 2002
Methylene blue	DcA-8	Dudley Chemical Co.	July 11, 2002
Orcein	ScOr-9	Sigma Chemical Co.	July 11, 2002
Resazurin	AcRz-26	Aldrich Chemical Co.	June 4, 2002
Wright stain	AcWr-90	Aldrich Chemical Co.	July 11, 2002

*Other information on these dyes will be furnished on request by the Biological Stain Commission, Department of Pathology, University of Rochester Medical Center, Rochester, NY 14642 Telephone: (585) 275-2751. Email bsclab@biostains.org. Website: www.biostains.org

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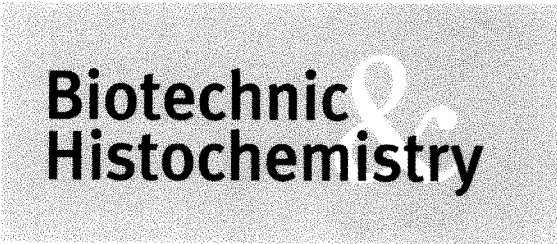
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